Modulation of the immune system by ligands of the C-type lectin DC-SIGN

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Modulation of the immune system by ligands of the C-type lectin DC-SIGN

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Voor mijn ouders

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Chapter 1

General Introduction

Adapted from:

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Introduction

The main task of the immune system is to eliminate life threatening entities (pathogens and tumors) while the normal tissues should be protected. To achieve this, the cells comprising the immune system developed several strategies to discriminate between self and non-self. A mechanism exploited by dendritic cells (DC) is based on the expression of Pattern Recognition Receptors (PRRs) [1,2]. A single PRR type has a preference for a given set of molecular patterns and when this preference is met, binding of this molecular pattern to a PRR will occur. Molecular patterns that bind to PRRs can be derived from either pathogens, from aberrant cells or normal cells. PRRs such as C-type lectin receptors (CLRs) and Toll like receptors (TLRs) (reviewed in [3]) bind to so called pathogen-associated molecular patterns (PAMPs), which include lipids, proteins and carbohydrates [1]. More specifically, CLRs recognize carbohydrate moieties present on pathogens, thereby playing an important role together with other PRR signals in the immune defense against pathogens [4,5]. In addition, CLRs recognize specific carbohydrates on apoptotic and malignant cells due to their altered glycosylation and can thus contribute to the clearance of abnormal cells [6,7]. Finally, CLRs are also involved in mediating cellular interactions between hematopoietic cells and plasma glycoprotein turnover [4]. CLRs bind carbohydrates via their carbohydrate recognition domains (CRDs) in a calcium (Ca^{2+})-dependent manner [8]. CRDs can be broadly divided into mannose- or galactose specific CRDs. The carbohydrate specificity is determined by the amino acid sequence comprising the CRD and on the ability and flexibility of the CRD to bind to a three-dimensional carbohydrate structure [4,9,10]. On the surface of microbes, these carbohydrate structures are typically found in dense arrays while on host cells the same or related carbohydrates are expressed in different configurations [11]. The mannose-binding lectin (MBL, also known as mannose binding protein), a soluble CLR circulating in the blood that upon binding to pathogen-derived carbohydrates initiate the complement cascade [12], specifically recognizes the characteristic orientation of sugars on pathogens whereas the glycan orientation on endogenous cells precludes tight multivalent interactions [4]. Nevertheless, other CLRs are more flexible in binding similar, but differently orientated, sugars exposed on pathogens and endogenous cells and are therefore able to recognize foreign as well as endogenous ligands [13,14]. For instance, dendritic cell-specific intercellular adhesion molecule-3 (ICAM-3) grabbing non-integrin (DC-SIGN, CD209), a CLR expressed by DC and certain macrophages, binds a broad range of ligands that include pathogen-derived ligands like human immunodeficiency virus-1 (HIV-1) [15], *Candida albicans* [16], and *Mycobacterium tuberculosis* [17], but also endogenous ligands like ICAM-2 [18] and ICAM–3 [19]. The ability of several CLRs to interact with both foreign as well as endogenous ligands indicates that a CLR alone is not sufficient to instruct the immune system to discriminate between self and non-self. TLRs are regarded to fulfill this role in providing the danger signal [20] since they recognize pathogen-derived ligands with an exquisite specificity. However, an increasing number of endogenous ligands for TLRs have been reported so far (reviewed in [21]). The discrimination between self and non-self is therefore not dependent on only one type of PRR, but rather on the interplay with multiple receptors which together will shape the immune response. Moreover, the immune system has several safety check points incorporated if one component fails to discriminate between self and non-self. For instance, after processing of trapped CLR ligands, these are presented in the context of major

histocompatibility complex (MHC) class II molecules to T cells for inspection. Moreover, regulatory T cells control immune reactions against self [22]. In this review, we will discuss the resulting responses upon binding pathogen-derived and endogenous ligands with regard to the CLRs expressed on DC. Moreover, general aspects of CLRs are discussed concerning their carbohydrate specificity, the relevance of combining multiple CRDs, the ability of several CLRs to signal or to interfere with other signaling pathways, and the interactions with other immune receptor families.

C-type lectins on DC

DC originate from the bone marrow and migrate to peripheral tissues where they become sessile as immature DC. These immature DC mainly function as endocytic cells and sample the environment for invading antigens by using its CLRs [23,24]. Upon inflammation, TLRs expressed on the DC will be triggered by specific PAMPs, leading to the maturation of the DC [25]. During this maturation step, a DC will acquire capacities to induce an adaptive immune response and lose its strong capacity to internalize pathogens. As a result, mature DC mainly function as antigen-presenting cells and are located in lymphoid organs to present specific antigens to naïve T cells [23]. In the absence of inflammation, no TLR signals are present and consequently the DC remain in their immature state and may play a role in tolerizing mechanisms such as maintenance of tissue homeostasis and the induction of regulatory T cells [26].
A wide variety of CLRs has been detected on DC that recognize carbohydrate structures on pathogens as well as on endogenous proteins. In general, most CLRs will function as PRRs by binding to and internalizing pathogens into lysosomal compartments for degradation to enhance antigen processing and presentation by the DC [27,28]. Since immature DC are strong phagocytes, they express a large diversity of CLRs. In general, CLR expression decreases upon maturation and this correlates with a decreased ability of mature DC to take up antigens [27]. Two major subsets of DC are described in humans. The myeloid subset includes interstitial DC and Langerhans cells that are strategically located in peripheral tissues at the site of entry of most pathogens. The other subset consists of the plasmacytoid DC that are located in lymphoid organs [29]. Immature interstitial DC express a considerable variety of CLRs including DC-SIGN, mannose receptor (MR) and DEC-205, whereas plasmacytoid DC and Langerhans cells express a limited number of CLRs like blood DC antigen-2 (BDCA-2) or Langerin respectively (reviewed in [27] and [30]).
Since CLRs are able to interact with carbohydrate structures on pathogens and on endogenous proteins, this indicates additional functions for CLRs next to their role in pathogen recognition. These functions include the establishment of homeostasis upon binding glycosylated endogenous ligands and the involvement in several cellular processes such as adhesion and migration [27]. Indeed, several CLRs such as DC-SIGN and MR have been implicated in mediating these functions upon binding endogenous proteins [13,27]. The MR is a CLR that contains eight CRDs and binds carbohydrates that have mannose, fucose or N-acetyl glucosamine as terminal sugar [31]. It is expressed on DC and macrophages, but also on lymphatic endothelium [32]. This CLR interacts with many pathogens such as *C. albicans* and HIV-1. This receptor was originally identified because it was able to recognize endogenous glycoproteins such as lysosomal hydrolases. MR mediates the clearance of these harmful agents,

thereby playing an important role in maintaining homeostasis [13]. Besides the clearance of endogenous ligands, MR expressed on lymphatic endothelium can also act as a cell adhesion molecule by binding to L-selectin on lymphocytes [32]. DC-SIGN is a CLR with only one CRD and, as described above, binds an extensive variety of viral, bacterial and parasitic ligands. Similar to the MR, DC-SIGN can act as an antigen-uptake receptor as well as an adhesion receptor by mediating DC-T cell and DC-endothelial cell interactions by binding to ICAM-3 and ICAM-2, respectively [18,19]. Recently, it was described that DC-SIGN mediates the interaction between DC and neutrophils through binding to Lewis X expressed by the β2-integrin MAC-1 of the neutrophil. The interaction between the two cell types induces the maturation of the DC and the immune response is skewed towards a T helper type 1 response [33]. This latter effect is a specific type of T cell response and responsible for cell-mediated/inflammatory immunity [34]. As potential clearance receptor, DC-SIGN is implicated in the binding of carcinoembryonic antigen (CEA) expressed on carcinoembryonic colorectal cancer tumor cells [7].

Structure of C-type lectins

Types of C-type lectins

Members of the CLR family contain one or more CRDs for binding to carbohydrate structures [30]. CLRs exist either as transmembrane proteins or as soluble proteins **(Fig. 1A).** Collectins such as MBL and the lung surfactant proteins A and D are examples of soluble CLRs and are important for the clearance of microorganisms (reviewed in [35]). Transmembrane CLRs can be divided into type I and II CLRs depending on the orientation of the amino terminus. Type I CLRs such as MR and DEC-205 contain several CRDs or CRD-like domains, and have an extracellular N-terminus. Type II CLRs comprising DC-SIGN, Langerin, dendritic cell immunoreceptor (DCIR), C-type lectin receptor 1 (CLEC-1), dendritic cell lectin (DLEC), BDCA-2, Dectin-1, and Dectin-2 have only one CRD and their N-terminus is intracellularly located [30]. For the type II CLRs, two main clusters can be identified in the human genome. One cluster is located at chromosome 19p13 and includes DC-SIGN, L-SIGN (liver/lymph node SIGN) and lymph node sinusoidal endothelial cell C-type lectin adjacent to CD23 and CD69 [36-38]. The second cluster maps at chromosome 12p13 and contains DCIR, CLEC-1, CLEC-2, Dectin-1, and DLEC, close to CD6, activation-induced C-type lectin (AICL), CD161, CD94 and the NKG2 family [39-42]. The localization into clusters suggests that the different functional CLRs descend from one ancestor gene during evolution to meet the need for more functional different CLRs [43].

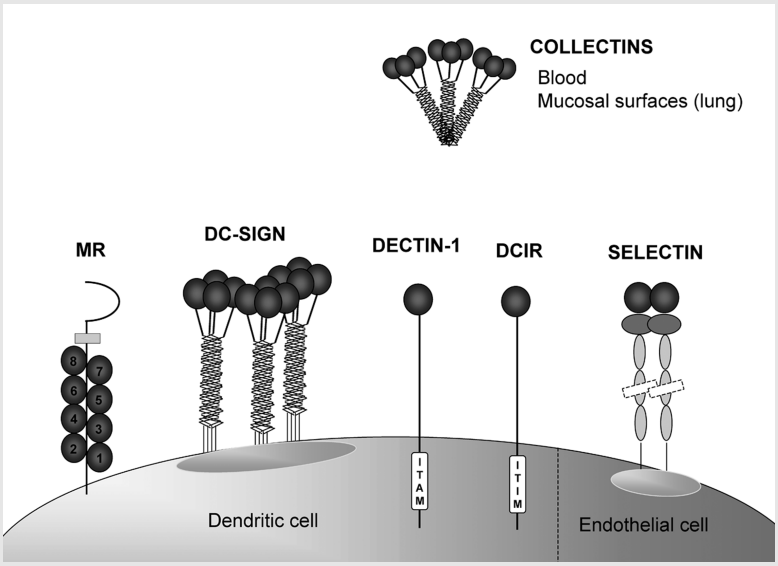
Carbohydrate recognition domain (CRD)

CLRs contain the prototype C-type lectin fold, consisting of two anti-parallel β strands and two α helices. This common fold contains irregular loop structures from which two are involved in monosaccharide binding [44]. Ca^{2+} is required in establishing the interaction of carbohydrates to the CRD and therefore these type of lectins are classified as C-type lectins. Carbohydrates interact with a primary ligand binding site in the CRD by coordinating bonds with the Ca^{2+} ion and further hydrogen bonding with amino acid side chains that also bind to the conserved Ca^{2+} [45]. The characteristic CRD can be subdivided into two

broad groups; those binding mannose-type carbohydrates and those binding galactose-type carbohydrates. The mannose-type CRD group contains the triplet amino acid sequence EPN, whereas the galactose-type CRD group contains the triplet QPD [46]. Each CRD recognizes a particular panel of carbohydrates that is, besides a specific sequence in the CRD domain, determined by the ability and flexibility of the CRD domain to bind multivalently to differently oriented sugars [9,10]. For example, DC-SIGN and its homologue L-SIGN bind mannose in a different manner compared to MBL. The CRD of MBL recognizes a single terminal mannose residue, and strong binding is only obtained when three CRDs are combined into a trimeric complex **(Fig. 1A)** [9]. The distance between individual CRDs within MBL oligomers is approximately 4.5-5.3 nm. Therefore, it has been proposed that a MBL can only recognize mannose densely packed on large mannosylated areas of pathogens and not the short distance (2-3 nm) between mannose residues present on endogenous proteins

Figure 1A

STRUCTURE AND ORIENTATION OF C-TYPE LECTINS. CLRs either exist as soluble or as transmembrane type I or type II lectins [30]. MR as well as DEC-205 (not shown in the Figure) are type I CLRs and contain multiple CRDs. MR contains eight CRDs of which CRD4 is most responsible for monosaccharide binding. However, multiple CRDs (4-8) are required for binding to multivalent ligands [68]. Another type I CLR is E-selectin which is an endothelial surface adhesion receptor for leukocytes. On these cells, E-selectin is located in lipid rafts and this localization is important for signaling during leukocyte-endothelial cell interactions [72]. The expression and function of E-selectin and other selectins on DC is still under investigation. DC-SIGN, Dectin-1 and DCIR belong to the type II CLR family and contain only one CRD. DC-SIGN forms a tetramer to increase its avidity for its ligands [9]. Moreover, DC-SIGN is located in microdomains (±200 nm in diameter) on the surface of DC which enhances the binding of virus-sized particles [70]. It is not known whether Dectin-1 and DCIR can form oligomers to increase their affinity for their ligands. Dectin-1 contains the activatory signaling motif ITAM in its cytoplasmic domain whereas DCIR contains the inhibitory signaling motif ITIM [41,77]. Collectins such as MBL and lung surfactant protein A and D are soluble CLRs and play an important role in innate immunity. They oligomerize to increase their affinity for carbohydrate ligands exposed on the surface of microbial pathogens [12].



[4,9]. In contrast, by forming tetramers, DC-SIGN and L-SIGN **(Fig. 1A)** can recognize mannose on pathogens as well as on endogenous proteins because they recognize other spatial arrangements [9]. Snyder *et al.* [47] developed a tetramer model by homology modelling and calculated that the surface area encompassed by the tetrameric CRD of DC-SIGN is approximately 16 nm^2 per CRD molecule. Ligands for DC-SIGN and L-SIGN must possess a surface glycosylation level exceeding one glycan molecule per 16 nm^2 of its surface area to allow multiple interactions with one tetramer. Several pathogen-derived as well as endogenous proteins contain this glycosylation level and are therefore ligands for DC-SIGN and L-SIGN [47].

Glycan arrays consist of an extensive panel of immobilized polysaccharides, glycoproteins, oligosaccharides and monosaccharides. Nowadays, these assays are frequently used as a new approach to investigate in more detail the carbohydrate preferences of several CLRs [48-50]. However, one has to be cautious with the analysis of these data because spatial arrangement plays a prominent role in binding. As an example of such studies, the binding specificity of DC-SIGN and L-SIGN was compared with a glycan array in which biotinylated oligosaccharides were immobilized in streptavidin-coated wells [51]. Fluorescently labeled extracellular domains of DC-SIGN and L-SIGN were probed to this array. Remarkable significant differences were observed for these two CLRs despite their 77% homology in amino acid sequence. The authors discovered that L-SIGN only binds mannose-containing glycans, while DC-SIGN is also able to bind fucose-containing glycans such as Lewis X [51]. Differences in the carbohydrate binding profile of DC-SIGN and L-SIGN were also demonstrated by van Liempt *et al.* [52], although they found that L-SIGN is able to bind to the fucose ligands Lewis A, B, and Y. In contrast to the afore-mentioned glycan array, van Liempt *et al.* used different Lewis antigens coupled to biotinylated polyacrylamide and tested the capacity of K-562 cells expressing DC-SIGN or L-SIGN for binding to these agents [52]. Probably, due to a rather low affinity for fucose ligands, binding is only established when L-SIGN is present in multiple copies on the surface of a cell. Both groups point to the amino acid Valine 351 present in the CRD of DC-SIGN that might explain the difference in fucose specificity [51,52]. Valine 351 in DC-SIGN creates a hydrophobic pocket that strongly interacts with the Fuc1,3/4-GlcNAc moiety of Lewis antigens. In L-SIGN, at the corresponding position of Valine 351 in DC-SIGN a Serine 363 is present and creates a hydrophilic pocket that excludes binding to Lewis X [52]. Since CRDs recognize subtle differences in the arrangement and branching of carbohydrates, a minor modification can already result in the loss of binding. For example, sialylation of Lewis X and ICAM-2 abrogates the binding to DC-SIGN [51,53]. Platelets with heavily sialylated ICAM-2 cannot bind to DC-SIGN, whereas endothelial cells with unsialylated ICAM-2 do bind DC-SIGN [53]. Thus, sialylation modulates the binding specificity of DC-SIGN. In addition, these subtle differences prevent the interference of DC-SIGN with other CLRs like the selectins that have a high specificity for sialyl Lewis X [51]. Nevertheless, some CLRs share an overlapping array of ligands and contribute to redundancy.

Cytoplasmic domain of CLRs

A general characteristic of most CLRs is that they endocytose foreign ligands that subsequently are targeted to lysosomal compartments. Peptide fragments that result from lysosomal degradation end up in MHC class II molecules at the cell surface and are in turn inspected by T cells for self/non-self discrimination. Endocytosis by CLRs that have bound ligand is mediated by conserved

Figure 1B

SIGNALING AND INTERNALIZATION MOTIFS ARE PRESENT IN THE CYTOPLASMIC TAILS OF CLRS. The cytoplasmic tails of CLRs contain several conserved motifs that may direct their intracellular targeting. Endocytosis is mediated by either di-leucine motifs (LL) and/or tyrosine-based sequences with the consensus motif FXXXXY or YXXØ (depicted in bold and bold underlined respectively; in which X designates any amino acid and Ø designates any amino acid with a bulky hydrophobic side chain). The motif YXXØ, a recognition site for adapter proteins that might mediate intracellular targeting [78], and a dileucine motif involved in targeting to the endosomal/lysosomal pathway [79] are found in the cytoplasmic tail of DC-SIGN. In particular, the dileucine motif has been shown to support internalization of DC-SIGN-ligand complexes in transfected cells [55]. This is in contrast to the MR and DEC-205, which are constitutively endocytosed through their tyrosine-based (FXXXXY) motifs [58]. Moreover, the cytoplasmic domain of DC-SIGN contains an acidic triad (EEE; in grey box) that in DEC-205 has been reported to target the CLR to organelles for antigen processing and peptide loading onto MHC class II molecules [58]. In addition, Dectin-1 and DCIR have an ITAM (depicted in bold in grey box) and an ITIM motif (depicted in bold cursive), respectively, which have been shown to induce specific signaling cascades [60,62].

DC-SIGN	MSDSKEPRLQQLGLLEEEQLRGLGFRQTRG**YKSL**AGC
L-SIGN	MSDSKEPRVQQLGLLEEDPTTSGIRLFPRDFQFQQIHGHK
DCIR	MTSE***ITYAEV***RFKNEFKSSGINTASSAASKERTAPLKSNTGFPK
BDCA-2	MVPEEEPQDREKG
Langerin	MTVEKEAPDAHFTVDKQNISLWPREPPPKSGPSLVPGKTPTVR
Dectin-1	ME**YHPDL**ENLDEDG**YTQL**HFDSQSNTRIAVVSEKGSCAASPPWR
DLEC	MVPEEEPQDREKGLWWFQLKVW
CLEC-2	MQDEDGYITLNIKTRKPALVSVGSASSSWWR
DCAL-1	MSEEVTYADLQFQNSSEMEKIPEIGKFGEKAPPAP
DEC-205	QRHRLHLAG**FSSVRY**AQGVNEDEIMLPSFHD
Endo180	RRRQNIERGA**FEGARY**SRSSSSPTEATEKNILVSDMEMNEQQE
MR	RRVHLPQEGA**FENTLY**FNSQSSFPGTSDMKDLVGNIEQNEHSVI

motifs in the cytoplasmic tails of these lectins such as the di-leucine motif and tyrosin-based sequences **(Fig. 1B)** [54]. DC-SIGN contains both motifs, but it appears that for this lectin only the di-leucine motif is required for internalization upon ligand binding since mutation of this di-leucine motif abrogated internalization [55]. The MR expresses only the tyrosin-based sequences [56]. Remarkably, MR recycles via early endosomes back to the cell surface enabling endocytosis of large quantities of glycoconjugates [57]. The tri-acidic cluster, expressed by DEC-205 and DC-SIGN, is a putative motif involved in routing internalized glycoconjugates to lysosomal and MHC-II-positive late endosomes indicating that these CLRs enable loading of peptides on MHC class II molecules [55,58]. Overall, it appears that cytoplasmic sequences determine the specific intracellular routing of each CLR. CLRs that express several different motifs like DC-SIGN seem to have specialized routing pathways that might depend on the nature of the bound pathogens [54].

Besides expression of internalization sequences in their cytoplasmic tails, some CLRs also contain signaling motifs like the immunoreceptor tyrosine-based activation motif (ITAM) or the immunoreceptor tyrosine-based inhibition motif (ITIM) **(Fig. 1B)**. The β-glucan receptor Dectin-1 contains an ITAM motif and cooperates with TLR-2 in eliciting inflammatory responses against zymosan [59]. Upon triggering this Dectin-1/TRL-2 pathway, the cytokines TNF-α and IL-12 are produced. Recently, it was discovered that Dectin-1 can also signal

via an additional pathway independent of TLR-2 [60]. In this pathway, the protein tyrosine kinase Syk is recruited to Dectin-1 in response to yeast, and this subsequently results in the production of IL-2 and IL-10. DC deficient for Syk are not able to produce these two cytokines, but are still able to produce IL-12 which indicates that Dectin-1/Syk and Dectin-1/TLR-2 pathways can operate independently [60]. Other CLRs that contain a complete or partial ITAM sequence are DC-SIGN, macrophage galactose-type C-type lectin (MGL), CLEC-1, and CLEC–2, but it is not clear yet whether these CLRs use a pathway similar to Dectin-1 [54]. DC-SIGN has been described to interfere with TLR-4 signaling upon *M. tuberculosis* binding, indicating the existence of a different or additional pathway [61].

DCIR contains an ITIM sequence and this sequence is able to inhibit B-cell receptor mediated Ca^{2+} mobilization and tyrosine phosphorylation [62]. However, the exact role of this motif in modulating DC function is still unknown. In addition to ITAM and ITIM sequences, most CLRs, like DC-SIGN and MR, contain multiple serine and threonine residues that are potential phosphorylation sites [54]. MR is indeed reported to be involved in signaling transduction events. For example, one study reported that the anti-human MR monoclonal antibody 19.2 inhibits the LPS-induced IL-12 production by DC by interfering with TLR signaling [63]. Another monoclonal antibody against MR induces phenotypic and functional maturation of immature DC that have the ability to dampen inflammation and to inhibit the generation of Th1-polarized immune responses [64]. Moreover, MR is implicated in the activation of NF-κB in response to *Pneumocystis carinii* [65]. The CLR BDCA-2 does not contain any signaling motif, however is able to induce Ca^{2+} mobilization [66]. It is conceivable that CLRs lacking signaling motifs might dynamically associate with other signaling proteins for example during microbial infection.

Multimerization

Recognition of carbohydrates by CLRs is highly dependent on the density of the carbohydrates present on the cell surface as well as on the degree of multimerization of the CRDs [67]. Multimerization of CRDs can occur within one single molecule like in MR **(Fig. 1A)**. MR contains several CRDs in its extracellular portion with only weak affinity for single carbohydrates. At least three of these CRDs must cooperate to achieve a high-affinity binding to multivalent glycoconjugates [68]. Multimerization of CRDs can also be achieved by oligomerization of several CLRs which is for instance observed for DC-SIGN [47]. DC-SIGN and L-SIGN both form tetramers when expressed at the cell surface as well as in a recombinant soluble form **(Fig. 1A)** [9,10]. The neck domain is responsible for this tetramer formation [9]. The portion of the neck domain adjacent to the CRD is sufficient to mediate formation of dimers, while regions near the N-terminus are required for stabilization of tetramers [10]. In general, multimerization of CRDs results in an increased binding potential for carbohydrates. This is exemplified by the finding that a monomeric DC-SIGN CRD is still able to bind the HIV-1 envelope glycoprotein gp120, but the avidity increases substantially when a tetrameric CRD is used [69]. Receptor oligomerization not only increases the avidity for oligosaccharides, but may also help to cluster cytoplasmic internalization motifs leading to a more efficient signaling platform for internalization [69]. It is very likely that the way of expression of CLRs on the cell surface will determine the degree of multimerisation. For instance, DC show a clustered distribution of DC-SIGN in so-called microdomains which are important for binding and internalization of virus

particles. A significant portion of these clustered DC-SIGN molecules resides in lipid rafts on DC [70]. Lipid rafts are microenvironments with elevated cholesterol and glycosphingolipid content with the property to include and exclude proteins to variable extents [71]. The localization of DC-SIGN in lipid rafts may create a platform that favors pathogen binding and bringing signaling molecules into contact with DC-SIGN that are recruited in the same rafts as well [67]. This localization into lipid rafts might be a common mechanism for more CLRs since E-selectin expressed on endothelial cells colocalizes with lipid rafts as well **(Fig. 1A)**. This localization seems to be important for signaling during leukocyte-endothelial cell interactions since E-selectin must associate and activate phospholipase Cγ, which will only take place when E-selectin is present in these lipid rafts [72]. Moreover, TLR-2 and TLR-4, which have been implicated to be involved in the actions of Dectin-1 and DC-SIGN respectively, were recently discovered to locate in lipid rafts as well [73,74]. After bacterial infection, TLR-2 is enriched in caveolin-1-associated lipid rafts at the surface of airway epithelial cells and is in this way involved in the initiation of the host response to potential bacterial infections [73]. Triantafilou *et al.* shows that TLR-4 is located in lipid rafts together with CD14 and other bacterial recognition immune receptors upon LPS stimulation [74]. They propose that different combinational associations of receptors within activation clusters determine the specific responses to a variety of bacterial stimuli [74,75]. The observation that certain CLRs and TLRs reside in lipid rafts together with the actual findings of interactions between two PRRs, like SIGN-related 1 (SIGNR-1) physically interacting with TLR-4-MD-2 [76] could possibly mean that these receptors reside in the same lipid rafts.

Glycoconjugates; binding partners of CLRs

General characteristics of glycoconjugates

Most organisms glycosylate an extensive part of their cell-surface and secreted proteins. In humans, about 80% of these proteins contain oligosaccharides (glycans). This high frequency of glycosylated proteins indicates a crucial function for protein glycosylation. In fact, it plays a role in stability of glycoproteins, protection against proteolytic cleavage, adhesion, antigenic variation and protective immunity [80,81].

Usually, glycans are found in nature as glycoconjugates which can be glycoproteins or glycolipids and greatly exceed the diversity of proteins and nucleic acids [82]. This diversity is the result of the different disaccharide linkages, anomerization of glycosidic linkages (α,β), branching of glycan chains, and modification of hydroxyl groups of sugars by various groups (phosphate-, sulphate-, amino-) in the glycan molecule [50]. In spite of the enormous variability, glycans can be divided in three major classes depending on how they are linked to proteins or lipids: N-linked glycans, O-linked glycans, and glycosylphosphatidylinositol (GPI) anchors [82].

N-linked glycans are the most abundant group in nature and consists of a pentasaccharide core structure that can be extended by up to five 'antennae' or branches. The oligosaccharide side chain is attached to the protein via an asparagine amino acid that is part of the Asn-X-Ser/Thr consensus sequence [80-82]. This consensus sequence is conserved between eukaryotes and prokaryotes [80]. Once the glycans are covalently attached to proteins, these are further modified in eukaryotes only. These modifications include

removal of several mannose residues and addition of sugar groups in the ER and the Golgi complex. As a result, eukaryotic N-linked oligosaccharides can be divided into high-mannose, complex, and hybrid type. Lower eukaryotes like yeast predominantly contain high-mannose structures, such as the DC-SIGN ligand mannan present on *C. albicans*, while higher eukaryotes like men have evolved their glycosylation machinery to produce more complex glycans [16,83,84]. In humans, N-linked structures are present on the vast majority of glycoproteins such as on the ICAM subfamily of adhesion molecules like the DC-SIGN ligands ICAM-2 and ICAM–3 [85]. Viral proteins often contain N-linked glycans like the envelope glycoprotein E2 from Hepatitis C virus (HCV) and the glycoprotein gp120 from HIV-1 [86-88]. Viruses use the host machinery to synthesize their glycoconjugates, and this results in glycoproteins that resemble the host glycoproteins, providing a manner for the virus to evade the immune system of the host [81,89]. Bacteria can also escape the human immune system by producing glycoconjugates that are closely related like the Lewis blood group antigens as found on for example *Helicobacter pylori* [84,90].

In contrast to the N-linked glycans, O-linked oligosaccharides are less frequently found on eukaryotic glycoproteins and do not contain a common core structure [81]. In these type of sugars, the carbohydrate residue is covalently attached to the amino acid side chains via the hydroxyl group of serine or threonine [82]. The process of O-linked glycosylation occurs on fully folded proteins in the Golgi complex in eukaryotes [80,81]. In bacteria, the O-glycosylation process takes place in the cytoplasm or at the interface between cytoplasm and surface appendages such as pili and flagella [80]. Many bacterial glycoconjugates have O-linkages while archaea contain predominantly N-linked glycans [91,92]. In humans, O-glycans are detected in mucins and collagens [82,89].

The third group of glycans, the GPI anchors attach proteins or glycoproteins to eukaryotic cell membranes. GPI anchors are naturally occurring glycolipids and share the same basic core structure with a linear tetrasaccharide attached to the 6-O-position of inositol [81,82]. The basic carbohydrate structure of GPI anchors in yeast and protozoan parasites is similar to those in mammals [84,90]. The existence of prokaryote GPI anchors is still a matter of debate [93].

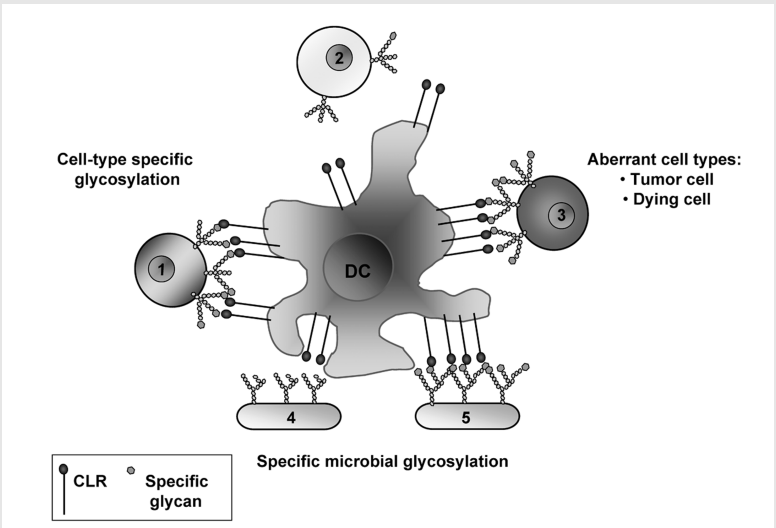
CLRs may specifically recognize one of these three groups of glycans, but the recognition of more than one group is also possible like MR in interacting with N-linked mannose structures present on HIV-1 as well as with O-linked mucins from normal and tumor cells [94-96].

Central to the different glycosylation processes described above are the glycosyltransferases. These enzymes catalyze the stepwise synthesis of both N- and O-linked oligosaccharides and can be grouped into many families like sialyl-, galactosyl-, and fucosyl-transferases, depending upon the type of sugar it transfers to the oligosaccharide chain [91]. The expression of human glycosyltransferases can be tissue specific which results in cells that present a unique set of oligosaccharides like the human blood group antigens Lewis A, B, X and Y [50]. Besides tissue-specific expression of glycosyltransferases, the differentiation status of the cell and the presence of cytokines can induce expression of certain glycosyltransferases resulting in the appearance of special glycans [50,97-99]. Tumor cells frequently express altered glycosylation patterns which include both the under- and over-expression of naturally-occurring glycans, as well as neoexpression of glycans normally restricted to embryonic tissues. These changes are normally the result of differences in the expression levels of

glycosyltransferases in the Golgi compartment of tumor cells [100]. The difference in glycosylation pattern of malignant tissue compared to healthy tissue results in a differential binding of lectins **(Fig. 2)** [101].
Microbes also express a unique set of glycans due to the presence of specific glycosyltransferases. This expression can be very dynamic to manage antigenic-diversity and immune evasion like in *H. pylori* **(Fig. 2)** and *Campylobacter jejuni*. In *H. pylori*, the dynamic expression of glycans is established by frame-

Figure 2

CLRS RECOGNIZE CARBOHYDRATE STRUCTURES EXPRESSED ON MICROBIAL AND ENDOGENOUS CELLS SUBJECT TO CELL-SPECIFIC OR MODIFIED GLYCOSYLATION. Several CLRs recognize endogenous cell-type specific glycosylation (1 vs. 2) such as DC-SIGN in interacting with neutrophils via Lewis X. This carbohydrate moiety is specifically expressed by Mac-1 on these type of cells [33]. Moreover, DC-SIGN recognizes ICAM-2 expressed on endothelial cells, but not the heavily sialylated ICAM-2 expressed by platelets [53]. Aberrant cells (3) such as dying and tumor cells frequently have an altered glycosylation compared to their original state. MBL has been reported to bind several malignant as well as apoptotic and necrotic cells (reviewed in [6]). In addition, DC-SIGN binds to breast and colorectal cancer cells and DEC-205 recognizes apoptotic thymocytes [7,102]. The interactions between CLRs and aberrant cells may result in clearance of these cells or may provide a way for tumor cells to escape immunity.
CLRs recognize specific strains of pathogens (4 vs. 5) due to their specific glycosylation. For instance, DC-SIGN binds to *M. tuberculosis* through the mannosylated mycobacterium cell wall lipoarabinomannan whereas *M. smegmatis* is not recognized because this structure is not mannose-capped [17]. Moreover, DC-SIGN specifically recognizes the Lewis+ variant of the human gastric pathogen *H. pylori* via the Lewis antigens expressed on LPS present on the bacterial surface. These Lewis antigens are dynamically expressed on *H. pylori* due to *on* and *off* switching of genes encoding for specific fucosyltransferases [103]. Interestingly, interaction between DC-SIGN and these two pathogens described above result in the modulation of the DC to escape immunity [17,103]. Dectin-1 specifically recognizes *C. albicans* yeast and not the filamentous form. This is accomplished by the exposure of the Dectin-1 ligand ß-glucan on bud scars in *C. albicans* yeast. In *C. albicans* filaments this budding does not occur and therefore no ß-glucan is exposed. Triggering Dectin-1 by *C. albicans* yeast results in a potent antifungal inflammatory response [104].



shift mutations and in *C. jejuni* by phase-variable genes encoding for enzymes involved in biosynthesis of carbohydrates [80,89].

Pathogen-derived glycoconjugates

The cell wall of pathogens is decorated with a wide array of carbohydrates. These are derived from genes of the pathogen itself, but also from genes of the host by horizontal transfer [1]. Especially viruses use this latter mechanism to escape the immune system by mimicking glycosylation of endogenous glycosylated proteins [85]. Moreover, enveloped viruses are also able to acquire host cell membrane glycoproteins. HIV-1 acquires ICAM-1, CD43, and many other cellular proteins when it buds from the infected host cells [105].

The main function of CLRs after binding pathogens is internalization, which leads to lysosomal degradation and subsequent loading of peptide fragments into MHC molecules **(Fig. 3)**. When this MHC-peptide complex is recognized by T cells, the adaptive immune system is activated [30]. However, several pathogens escape the immune system by exploiting specific CLRs. For instance, HIV-1 binds to DC-SIGN on DC at the site of infection and exploits the migratory capacity of DC to gain access to T cells in the lymph node [15]. Also other viruses target DC-SIGN to promote their dissemination like HCV [106] and severe acute respiratory syndrome coronavirus [107]. HIV-1 and HCV are internalized after binding to DC-SIGN **(Fig. 3)** into the DC, but instead of undergoing lysosomal degradation these virus particles reroute from endosomes to the cell surface and subsequently infect T cells *in trans* [106,108]. In addition to DC-SIGN, the MR on macrophages is also exploited by HIV-1 for transmission to permissive T cells. The half-life of HIV-1 bound to MR is lower compared to virus bound to DC-SIGN, indicating different internalization routes for MR and DC-SIGN [96].

Besides targeting of CLRs by viruses for transmission to permissive cells, non-viral pathogens exploit CLRs for immune escape in other ways. *M. tuberculosis* targets DC-SIGN and causes the inhibition of the immunostimulatory function of the DC by producing the immune response dampening cytokine IL-10 [17]. The human gastric pathogen *H. pylori* also modulates the function of DC via DC-SIGN by blocking the polarization towards a T helper type 1 response [103]. Another CLR that might be exploited by pathogens to modulate the DC function is BDCA-2. This CLR is specifically expressed by plasmacytoid DC and is able to change the direction of the immune system by mediating the production of specific cytokines [109].

Despite the various cases of immune evasion by pathogens via CLRs, CLRs still play an important role in limiting the spread of infectious organisms. Mice lacking SIGN-R1, the murine homologue of DC-SIGN, fail to clear *Streptococcus pneumoniae* from the circulation and are therefore more susceptible to infection with this pathogen [110]. MBL-null mice die 48 hours after exposure to *Staphylococcus aureus* underlining the importance of MBL in restricting the complications when infected with this pathogen [111]. A complete comparison between these mouse disease models and infectious diseases in man is difficult since remarkable differences exist between human DC-SIGN and mouse SIGN-R1 with respect to cell type specific expression patterns (reviewed in [112]).

Next to CLR binding, a pathogen also interacts with other receptors present on the DC, such as members of the TLR family. These TLRs recognize characteristic patterns on specific pathogens. For instance, TLR-4 recognizes LPS on Gram-negative bacteria [3]. The engagement of a specific TLR with associated signaling complexes results in an appropriate response against the pathogen involved

[59]. CLRs can cooperate with these receptors, like Dectin-1 does with TLR-2, but can also interfere, like DC-SIGN does with TLR-4 [17,60,113]. Recently, it was reported that SIGN-R1 associates with TLR-4 to capture gram-negative bacteria and to facilitate signal transduction to activate innate macrophage responses [76]. Apparently, in this kind of response SIGN-R1 cooperates with TLR-4 instead of interfering like observed for the human DC-SIGN.
Several CLRs can interact with more than just one pathogen as exemplified for DC-SIGN and MR [5,114]. The multiple engagement of a pathogen with a CLR in combination with other molecules expressed on the DC will result in a unique response. The response is also dependent on the structure of the CLR ligand. To illustrate this, the DC-SIGN ligands Lewis X antigen and HCV show different internalization routes in immature DC. The Lewis X antigen is internalized into lysosomes upon binding DC-SIGN, whereas HCV virus-like particles are targeted to nonlysosomal compartments [106]. Similar to HCV, HIV-1 binds the same binding site on DC-SIGN and is also targeted to nonlysosomal compartments via DC-SIGN [15,106].

Endogenous glycoconjugates

Carbohydrate structures that decorate proteins and lipids on the cell surface are representatives of normal self. These carbohydrate structures usually terminate with sialic acids in vertebrates. Those sialic acids are recognized by a variety of receptors involved in intercellular communication like the Siglecs group [1]. The Siglecs group contain ITIM motifs in their cytoplasmic tail which indicates that they negatively regulate the function of leukocytes upon binding sialic acids [115]. For instance, CD33 a member of the Siglec family is expressed on monocytes and constitutively represses monocyte activation due to interactions with sialic acids residues [116]. Lack of sialic acid on most micro-organisms and on transformed cells is recognized as missing self, thus overcoming the inhibitory effect of the Siglecs [1]. Several studies indicate a tolerizing function for CLRs upon binding endogenous ligands. For instance, the MR mediates tissue homeostasis and resolution of inflammation by the clearance of potential harmful endogenous products [4,13,114]. This function is also ascribed to MGL since it has a specificity for terminal α- and β-linked GalNAc residues that naturally occur as parts of glycoproteins or glycosphingolipids [117]. Moreover, Bonifaz *et al.* show that ovalbumin protein targeting to DEC-205 in mice results in tolerance induction, when no danger signal is present [118].
The *in vivo* localisation of CLRs in peripheral tissues also points to a tolerance inducing function for several CLRs. In the placenta, expression of DC-SIGN and MR is observed on decidual immature DC and macrophages whereas L-SIGN is detected on endothelial cells [95,119-121]. The presence of these CLRs in the placenta suggests that these CLRs play a role in the balance between defense against pathogens and tolerance of the fetal allograft [95,122]. Moreover, expression of DC-SIGN, L-SIGN and MR is detected on specific cells in the brain, an immunologically privileged organ [123,124]. This implies a role for these CLRs in limiting the spread of inflammation in the brain in order to avoid threatening of organ integrity and function [125,126]. Besides endothelial cells in the placenta and brain, L-SIGN is also expressed on liver sinusoidal endothelial cells which are tolerogenic organ-resident antigen-presenting cells [127].
Several CLRs are involved in cell-cell adhesion processes. DC-SIGN, Dectin-1, Dectin-2, and Dendritic Cell Associated Lectin (DCAL) mediate DC-T cell interactions [19,77,128,129]. These interactions result either in augmentation of T cell proliferation, as in the case of Dectin-1 [77] and DCAL [129], or in

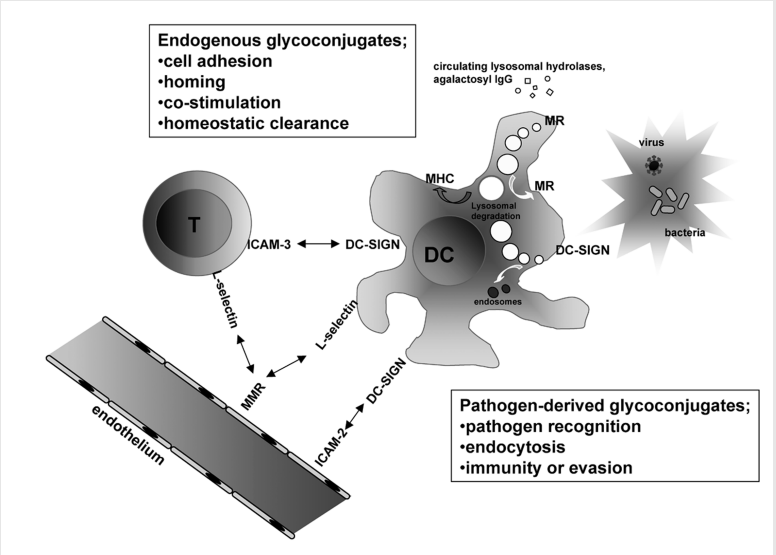
abrogation of the immune response as observed for Dectin-2 [128]. Dectin-1 and DCAL His-tagged fusion proteins deliver costimulatory signals to T cells in the presence of anti-CD3 antibodies [77,129]. In rat, Dectin-2 binds to regulatory T cells and mediates hapten-specific tolerance which is induced by UV radiation [128]. DC-SIGN **(Fig. 3)** is able to perform a stimulatory as well as an inhibitory role in DC-T cell interactions depending on the strength of the T cell stimulus [19,130].

Besides DC-T cell interactions, DC-SIGN mediates DC migration across the endothelium by binding to ICAM-2 **(Fig. 3)** [18]. For MR a trafficking function has been proposed by binding to sulphated carbohydrate ligands such as sulfo-Lewis A and X sequences. The recognition of sulfo-Lewis A and X sequences overlaps with the binding specificity of selectins, a CLR family specialized in leukocyte trafficking [131,132].

As mentioned previously, DC-SIGN is also involved in mediating interactions between DC and neutrophils. This interaction is established by DC-SIGN binding to Lewis X expressed on the β2-integrin Mac-1. In contrast to most of the endogenous ligands, this interaction takes place during an inflammation when neutrophils infiltrate inflamed tissue where they meet DC. The interaction results in a modulation of the immune response [33]. Interestingly, only

Figure 3

CLRS EXPRESSED ON DC INVOLVED IN RECOGNITION OF PATHOGEN-DERIVED AND ENDOGENOUS GLYCOCONJUGATES. DC-SIGN and MR function as PRRs by internalizing pathogens that are subsequently processed and loaded into MHC molecules for antigen-presentation. To take up large quantities of antigen, the MR recycles back to the cell surface via early endosomes [57]. Several pathogens exploit CLRs to escape immunity. HIV-1 and HCV exploit DC-SIGN to escape from the lysosomal degradation pathway remaining alive in endosomal compartments [106,108]. Moreover, the non-viral pathogens *H. Pylori* and *M. tuberculosis* escape immunity by modulating DC to produce anti-inflammatory cytokines upon binding DC-SIGN [17,103]. Besides functioning as PRRs, DC-SIGN and MR also function as adhesion and homing receptors by mediating contacts between DC and T cells (DC-SIGN), DC and endothelium (DC-SIGN) and T cells and endothelium (MR) [18,19,32]. As clearance receptor, MR internalizes lysosomal hydrolases and agalactosyl IgG to mediate homeostasis [13,134].



Mac-1 expressed by neutrophils contain the Lewis X structure and not when expressed by other immune cells like T cells **(Fig. 2)** [33]. This emphasizes the specificity of glycan expression on special cells and function of CLRs. Dying as well as malignant cells frequently have an altered glycosylation that results in the modification of glycans marking them as altered self **(Fig. 2)**. These modified self carbohydrates are detected by several CLRs like DEC-205, which participates in the clearance of apoptotic thymocytes, or MBL, which binds to apoptotic cell lines [6,102]. The clearance of apoptotic cells prevents the release of inflammatory intracellular components that are normally hidden inside the cells [1]. In addition, MBL can bind to carcinoma cell lines thereby mediating the clearance of these cells in a non-inflammatory way [6]. In analogy, DC-SIGN can bind colorectal cancer cells and may provide a way for these tumor cells to escape from immunological attack [7].
The endogenous ligands for DC-SIGN appear to be predominantly cell-associated, but recently a soluble ligand for DC-SIGN in the female genital tract was discovered. This naturally occurring glycoprotein is observed in 12.6% of the women studied and may provide innate protection against HIV-1 infection [133]. As mentioned previously, MR also binds soluble ligands **(Fig. 3)** such as lysosomal hydrolases to prevent tissue damage [13]. Moreover, MR can also bind agalactosyl immunoglobulins (IgG) which are increased in autoimmune disorders [134].

Concluding remarks

CLRs were primarily considered to be PRRs in recognizing glycosylated ligands on pathogens. However, this view is changing since many endogenous glycoconjugates are being discovered as ligands for the CLRs. The functional outcome upon interaction with a CLR, depends on the interplay with other antigen-sensing receptors like TLRs. The balance between CLRs and TLRs is critical for the type of immune response, resulting in either immune escape or complete elimination of a specific pathogen. Glycosylation differences on microbes and endogenous proteins can have profound impacts on the binding capacity of a CLR. With the use of glycan arrays the exact binding epitopes can be determined and will provide more information regarding the conditions a CLR will bind to its ligand. Unraveling the specific signaling pathways and interactions with other ligand recognition systems will be essential for a full understanding of the CLR biology. This knowledge about the CLR biology can be applied in therapeutic settings like in antigen targeting to CLRs [135] to either evoke an immune response in the case of cancer or dampen an immune response in the case of autoimmunity.

Aim of this thesis

DC-SIGN is a C-type lectin specifically expressed on DC that recognizes high-mannose and fucose glycans. This carbohydrate specificity of DC-SIGN enables recognition of a large array of ligands which include both pathogen-derived as well as endogenous glycoconjugates.
Because of the wide expression of DC-SIGN ligands, DC-SIGN is involved in several aspects of the immune system like immune defense, tolerance induction and cell-cell communication. This broadens the role of DC-SIGN since C-type lectin receptors were primarily considered as pattern-recognition receptors that recognize pathogen-associated molecular patterns. Therefore, the aim of this thesis was to investigate the various implications of DC-SIGN in interacting with foreign and endogenous ligands.
In **Chapter 2** and **3** the pathogenic DC-SIGN ligand *Candida albicans* was examined. The fungus *C. albicans* was discovered as a ligand for DC-SIGN in **Chapter 2**. DC-SIGN expressed on transfected cell lines and on monocyte-derived DC was able to bind the yeast form of *C. albicans*. After binding, the fungi were internalized into DC-SIGN enriched vesicles that may lead to antigen presentation. In **Chapter 3** the interaction of DC with *C. albicans* was further studied. We compared the anticandidal properties of DC with monocytes and macrophages and observed that DC were significantly less efficient in killing *C. albicans*. Moreover, DC released less pro-inflammatory cytokines in response to this fungus compared to monocytes and macrophages underlining the role of DC in antigen presentation. In **Chapter 4, 5** and **6** the endogenous ligands of DC-SIGN are described. The interaction of DC-SIGN with ICAM-3 was analyzed in **Chapter 4** to study the relevance of DC-SIGN in DC-T cell communication. DC-SIGN mediates DC-induced T cell proliferation, but this effect was only visible when the MLR is weak and the number of DC-SIGN binding PBL is high. In **Chapter 5** the interaction between DC-SIGN and monocytes via Lewis X is shown. This interaction depends on the density of Lewis X molecules expressed by monocytes. The density was modified by uncovering hidden Lewis X residues by removing sialic acid residues. In **Chapter 6** the binding capacity of DC-SIGN and L-SIGN to leukemic cells is described and this lead to the discovery that a high binding of DC-SIGN/L-SIGN to peripheral blood cells is correlated with a poor survival. Finally, in **Chapter 7** the potential of using DC-SIGN as a target to deliver antigens to dendritic cells is explored. In this chapter the effectiveness of an anti-DC-SIGN antibody coupled to KLH (hD1-KLH) to target DC and to induce an effective immune response against KLH is investigated. hD1-KLH specifically bound DC-SIGN, was internalized into the lysosomal compartment of DC and induced T cell proliferation at a 100-fold lower concentration than KLH alone.

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Chapter 2

The C-type lectin DC-SIGN (CD209) is an antigen-uptake receptor for *Candida albicans* on dendritic cells

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Abstract

Dendritic cells (DC) that express the type II C-type lectin DC-SIGN (CD209) are located in the submucosa of tissues, where they mediate HIV-1 entry. Interestingly, the pathogen *Candida albicans,* the major cause of hospital-acquired fungal infections, penetrates at similar submucosal sites. Here we demonstrate that DC-SIGN is able to bind *C. albicans* both in DC-SIGN-transfected cell lines and in human monocyte-derived DC. The binding was shown to be time- as well as concentration-dependent, and live as well as heat-inactivated *C. albicans* were bound to the same extent. Moreover, in immature DC, DC-SIGN was able to internalize *C. albicans* in specific DC-SIGN-enriched vesicles, distinct from those containing the mannose receptor, the other known *C. albicans* receptor expressed by DC. Together, these results demonstrate that DC-SIGN is an exquisite pathogen-uptake receptor that not only captures viruses but also fungi.

Introduction

Epithelial surfaces form the first line of defense against microbes. A small proportion of incoming microbes that enter at sites of microlesions is handled by APC, in particular DC [1]. Among pathogens that invade mucosal surfaces, *Candida albicans* is among the most frequently isolated from humans [2]. As *C. albicans* is the major cause of hospital-acquired fungal infections [3], its recognition by cell surface receptors has major pathogenetic consequences. Nevertheless, only limited information is available on the molecular mechanisms involved in recognition of this fungus. *C. albicans* can switch from a unicellular yeast form into various filamentous forms, all of which can be found in infected tissues [4]. The ability to reversibly switch between these forms is thought to be important for *C. albicans* virulence. Several studies now demonstrate that DC can bind and phagocytose fungi such as *C. albicans* [5-7]. Protection from mucocutaneous candidiasis clearly relies on cell-mediated immunity induced after DC process *C. albicans* and then present antigens that prime T cells [8].

Recent studies in mice demonstrate that whereas the yeast-form activates DC to produce IL-12 and primes Th1 cells, the hyphal-form inhibits IL-12 and Th1 priming and induces IL-4 production [5]. These results indicate that DC fulfill the requirement of a cell uniquely capable of sensing the two forms of *C. albicans* [5]. In addition, it was recently reported that human DC are also able to bind *C. albicans*, and that this interaction is mediated by the mannose receptor (MR, CD206), which is also found on macrophages [8].

However, the observation that *C. albicans,* both as a commensal as well as a true pathogen, is also found in areas (sub-mucosa) highly enriched in DC-specific ICAM-3 grabbing non-integrin (DC-SIGN)-positive DC prompted us to investigate whether *C. albicans* could be bound by DC-SIGN as well. Recently, we isolated this novel C-type lectin (also designated CD209) from monocyte-derived DC. We discovered that DC-SIGN acts as a binding partner for ICAM-3, mediating the early contact between DC and T cell and therefore the initiation of primary immune responses [9]. In addition, DC-SIGN displays a high affinity for ICAM-2, supporting transendothelial migration of DC and DC trafficking [10]. Moreover, DC-SIGN binds and captures HIV-1 at mucosal sites of initial infection, and protects the virus from degradation for subsequent transport by DC to lymphoid organs [11].

Here we demonstrate for the first time that as well as viruses, fungi are also recognized by DC-SIGN.

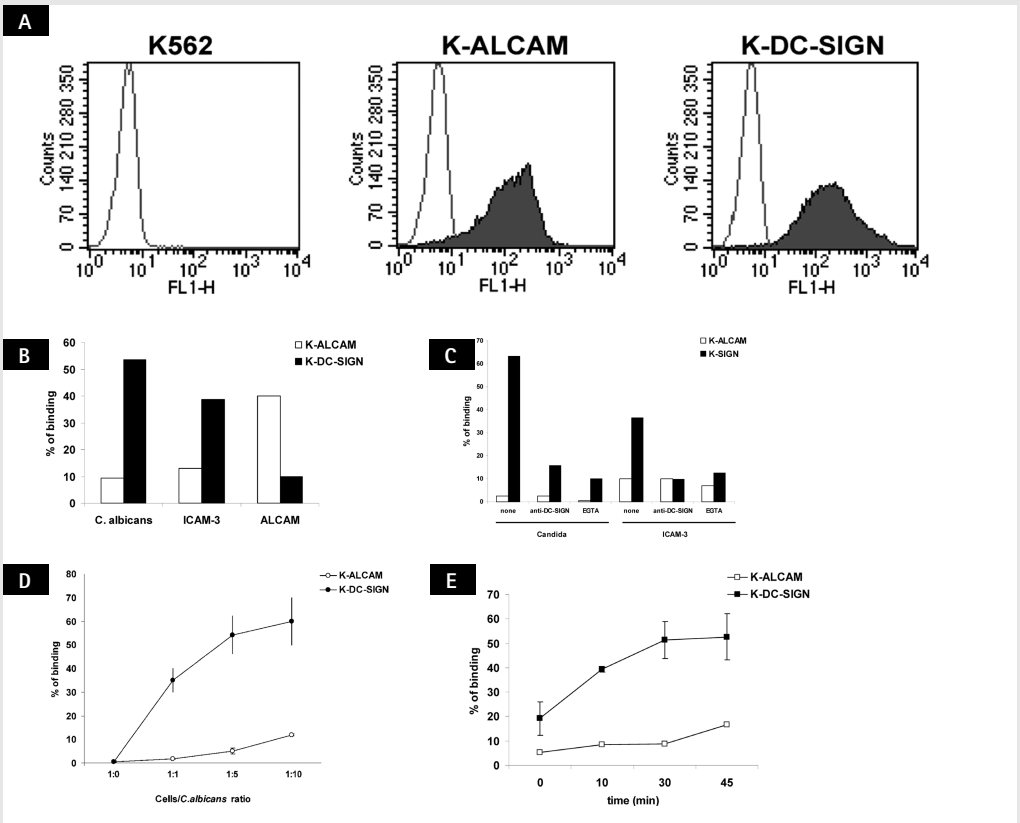
Results and Discussion

Candida albicans is a ligand of DC-SIGN

We used the erythroleukemic cell line K562 transfectants stably expressing DC-SIGN (K-DC-SIGN) [9] to investigate the potential of *C. albicans* to bind DC-SIGN in the absence of any other known *C. albicans* receptors. Binding to ICAM-3-coated fluorescent beads was used as a positive control for DC-SIGN function. K562 cells (K-ALCAM) transfected with the homotypic activated-leukocyte cell adhesion molecule (ALCAM) [12], which is expressed by DC but does not bind any of the known ligand of DC-SIGN, were used as negative control. The K562 transfectants stably express DC-SIGN and ALCAM **(Fig. 1A)** with ex-

Figure 1

DC-SIGN SPECIFICALLY BINDS THE YEAST FORM OF *C. ALBICANS.* (A) Transfectants (K562) stably expressing DC-SIGN or ALCAM were used for binding studies. The mAb AZN-D1 and AZN-L50 were used to detect DC-SIGN and ALCAM, respectively; isotype-matched Ab was used as control. (B) *C. albicans* binds to DC-SIGN but not to ALCAM: transfectants were labeled with CD45-allophycocyanin to discriminate cells binding FITC-labeled yeast from yeast aggregates. A representative experiment out of five is shown. (C) *C. albicans*– and ICAM-3-specific adhesion was determined in the presence of blocking anti-DC-SIGN mAb (20 µg/ml). The addition of EGTA (5mM) showed that DC-SIGN-mediated binding is Ca^{2+} dependent. One representative experiment out of three is shown. (D) *C. albicans* binding to DC-SIGN increases with increasing *Candida*:cell ratio. Aliquots of $50x10^3$ DC were incubated with various concentrations of heat-killed *Candida* cells for 30 min at 37°C. (E) Binding of *C. albicans* increases over time. One of two experiments is shown.



pression levels similar to those observed for immature DC [9;12].
Binding studies demonstrated that DC-SIGN clearly mediates adhesion to both *C. albicans* and ICAM-3-Fc-coated beads **(Fig. 1B)**. In contrast, K-ALCAM cells bound neither ICAM-3 nor *C. albicans*, but did bind ALCAM-Fc beads. Blocking antibodies against DC-SIGN significantly inhibited binding of *C. albicans* by K-DC-SIGN; in addition, the calcium chelator EGTA completely abrogated binding **(Fig. 1C)**. This Ca^{2+} dependence confirms that the C-type lectin domain of DC-SIGN mediates binding to *C. albicans*. With increasing concentrations of *C. albicans* **(Fig. 1D)** and increasing incubation time **(Fig. 1E),** binding of K-DC-SIGN to the yeast cells increased significantly.

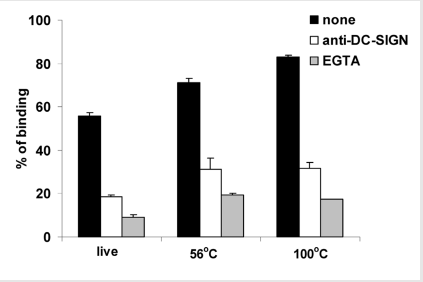
DC-SIGN binds both live and heat-inactivated yeast forms of *C. albicans*

To exclude the possibility that the binding of DC-SIGN to heat-inactivated *C. albicans* was due to artifacts derived from the heat treatment, K-DC-SIGN were allowed to interact with both live and heat-inactivated (see Materials and Methods) *C. albicans* yeast. As shown in **Fig. 2**, the percentage of binding did not increase significantly upon heat inactivation of the yeast, when compared with binding to live yeast cells. In addition, the blocking of binding by Ab against DC-SIGN was not profoundly altered by heat treatment.

Monocyte-derived DC also bind *C. albicans* through DC-SIGN

DC are specialized in binding and uptake of antigen [13] and recently it has been shown that the interaction between DC and *C. albicans* is mediated by the MR [5;8]. Our findings with K-DC-SIGN cells and the observation that *C. albicans* can be found in areas of the body (sub-mucosa) that are highly enriched in DC-SIGN-positive cells [11] suggested that DC-SIGN could contribute to the binding of *C. albicans* to immature DC.
In **Fig. 3A**, it is shown that human monocyte-derived immature DC are able to bind *C. albicans*, and that this interaction increases with time. This is in agreement with previously published work showing that DC rapidly internalize *C. albicans*: within 10-20 min of incubation at 37°C, already 40-50% of the particles are ingested, reaching a maximum after 60 min [5;8].
To determine the contribution of both MR and DC-SIGN to mediate binding of *C. albicans*, immature DC were incubated with specific inhibitors before interacting with the yeast particles. As shown in **Fig. 3B**, antibodies against DC-SIGN significantly blocked binding, though only partially (approximately 25-30%),

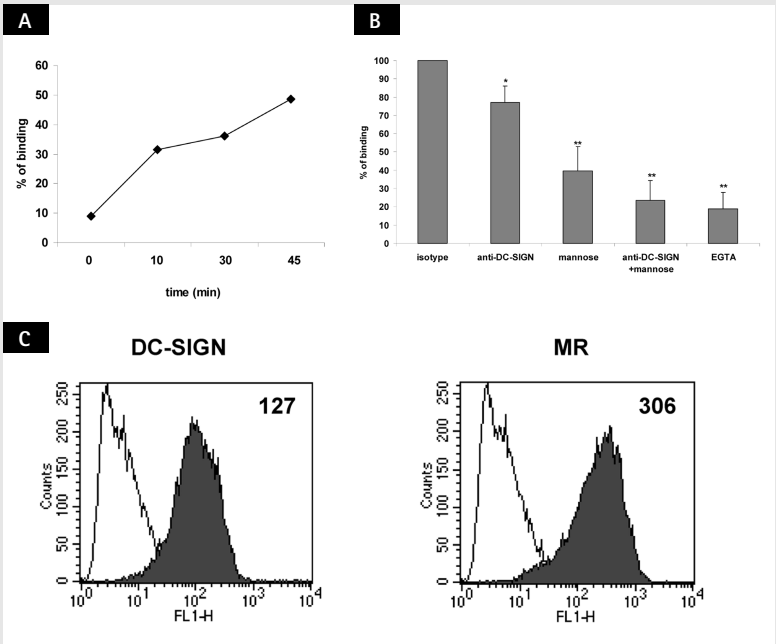
Figure 2



LIVE OR HEAT-KILLED YEAST FORMS OF *C. ALBICANS* ARE BOUND BY DC-SIGN. CD45-allophycocyanin-labeled K-DC-SIGN transfectants ($50x10^3$) were incubated for 30 min at 37°C with FITC-labeled *C. albicans* ($500X10^3$), either live or heat-inactivated (at 56°C or 100°C). A representative experiment out of two is shown.

Figure 3

DC BIND *C. ALBICANS* ALSO THROUGH DC-SIGN. (A) Binding of immature DC to *C. albicans* increased over time. CD45-allophycocyanin-labeled DC (50 x 10^3) were incubated with FITC-labeled heat-inactivated *C. albicans* (500 x 10^3). One representative experiment out of two is shown. (B) Immature DC bind *C. albicans* through C-type lectins. CD45-allophycocyanin-labeled DC (50 x 10^3) were incubated with FITC-labeled heat-inactivated *Candida* (500 x 10^3) in the absence or presence of anti-DC-SIGN mAb (20 µg/ml), mannose (100 mM), a mixture of anti-DC-SIGN Ab (20 µg/ml) and mannose (100 mM), or EGTA (5 mM). The average of six independent experiments is shown. The binding to *C. albicans* in absence of inhibitors (with isotype-matched control) was set as 100%. The significance levels (*p<0.01 and **p< 0.001) derive from comparing the percentage of binding in the presence of blocking agent versus the percentage of binding in absence of blocking agent. (C) FACS profile of immature DC expressing MR and DC-SIGN. mAb clones 19.2 and AZN-D1 were used to label MR and DC-SIGN, respectively. Cells were gated on forward-side scatter, and the mean fluorescence is shown in the top right corner of the histograms.



whereas mannose, which is known to specifically inhibit the MR, was responsible for blocking about 65-70% of the binding. Interestingly, mannose did not show any significant blocking of *C. albicans* binding to DC-SIGN on K-DC-SIGN cells (not shown). Moreover, by combination of both anti-DC-SIGN Ab and mannose, binding of *C. albicans* was almost completely inhibited (80-85%). This notion is supported by the observation that EGTA blocked binding of *C. albicans* to DC to a similar level, also indicating that probably no other C-type lectins were involved. Furthermore, the finding that binding of *C. albicans* was mediated by DC-SIGN and MR, in a ratio of roughly 1:3, respectively, was in agreement with the observation that the expression of MR on the surface of immature DC appears to be higher than that of DC-SIGN **(Fig. 3C).** However, no real quantitative evaluation can be made.

In addition, besides DC-SIGN and the MR, DC are known to express high levels of the $ß_2$-integrin MAC-1, which has already been implicated in the binding of *C. albicans* to lymphocytes [6]. However, we could not detect any blocking when anti-$ß_2$-integrin antibody (NKI-L19) was used, suggesting that MAC-1 is not likely to be involved in *C. albicans* binding on human immature monocyte-derived DC (data not shown). Moreover, the use of laminarin, reported to interfere with the interaction between another DC-specific C-type lectin, Dectin-1, and *C. albicans*, did not show any blocking effect either (data not shown). Together, these findings strongly suggest that binding of *C. albicans* to immature DC is predominantly mediated by the C-type lectins DC-SIGN and MR.

DC-SIGN mediates phagocytosis of *C. albicans*

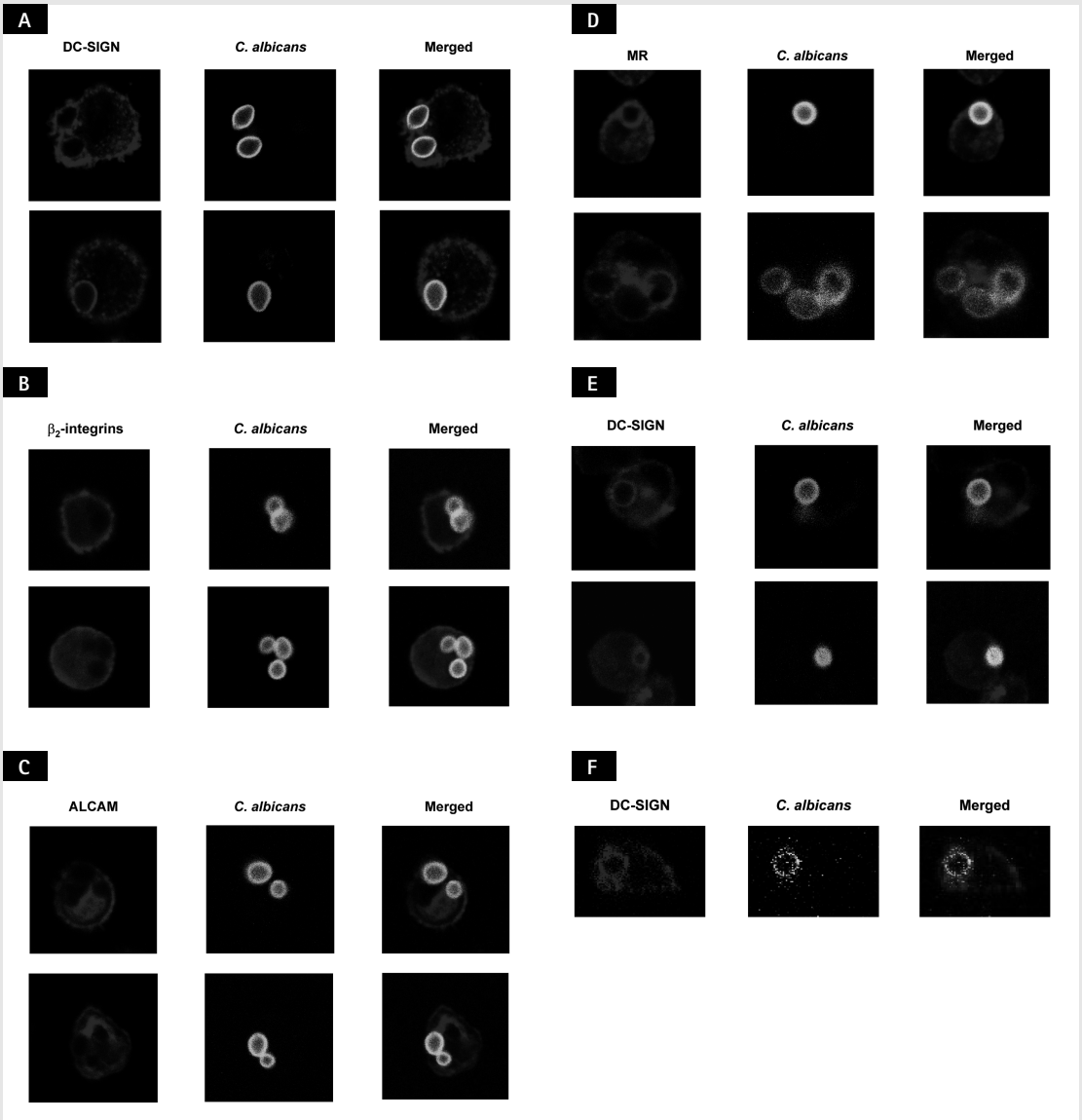
We recently demonstrated that DC-SIGN can act as an antigen-uptake receptor and facilitates phagocytosis within minutes [14]. To determine whether DC-SIGN could contribute also to the internalization of *C. albicans* by immature DC, we incubated DC with FITC-labeled *C. albicans* particles for 60 min at 37°C to allow phagocytosis. Subsequently, we fixed, permeabilized, and fluorescently labeled DC with specific Ab against various receptors, and analyzed by confocal microscopy.

In general, we observed that after 1 h of incubation, about 40% of immature DC have ingested *C. albicans*, ranging from 1 to 9 particles (average of 3) per DC. The results in **Fig. 4A** show that DC-SIGN clearly co-localizes with *C. albicans* particles, indicating the involvement of this lectin in binding and uptake of this pathogen. By contrast, neither MAC-1 nor ALCAM were found to localize around the yeast particles **(Fig. 4B, C)**.

Fig. 4D shows colocalization between MR and ingested *Candida*. In order to determine whether DC-SIGN was also able to internalize *C. albicans* in presence of inhibitors of MR, DC were allowed to phagocytose the yeast particles in presence of mannose. As shown in **Fig. 4E**, vesicles containing DC-SIGN colocalizing with FITC-labeled *Candida* can still be clearly observed. To prove that *C. albicans* were indeed ingested, we made Z-scans, unequivocally demonstrating that the yeast particles are ingested **(Fig. 4F).** The presence of EGTA almost completely blocked phagocytosis (data not shown).

Figure 4

DC-SIGN MEDIATES PHAGOCYTOSIS OF *C. ALBICANS* IN DC. Immature DC were incubated with *C. albicans* yeasts at a DC:*C. albicans* ratio of 1:5 for 1 h at 37°C to allow phagocytosis. Subsequently, samples were fixed and labeled for confocal microscopy: the images show FITC-labeled *C. albicans* (green), Cy5-labeled adhesion receptors on immature DC (blue) and colocalization (merged) (see Materials and Methods). (A) Colocalization of DC-SIGN with FITC-labeled *C. albicans* was clearly observed. Labeling of β_2-integrins (B) as well as ALCAM (C) showed no colocalization in the vesicles containing the pathogen. (D) Labeling of MR, the other receptor for *C. albicans* on immature DC, showed considerable colocalization with the yeast. In addition, DC-SIGN-enriched vesicles were observed also in presence of 100 mM mannose, which inhibits the MR (E). The Z-scan (F) indicates that the yeast particles were indeed ingested by the DC.



Conclusions

We showed that as well as viruses (HIV-1, SIV, Ebola) [11;15;16] and parasites (Leishmania) [17], the C-type lectin DC-SIGN can also bind yeast (*C. albicans*). These observations, together with the discovery that DC-SIGN acts as an antigen-uptake receptor, provide further evidence that C-type lectins on DC are major pathogen-recognition receptors [18]. Distinct from the Toll like receptors [19], they mediate antigen uptake rather than activating DC.

Our findings clearly show that *C. albicans* has two major receptors on human monocyte-derived DC; DC-SIGN and MR. DC-SIGN is expressed at sites in the skin (dermis) and the mucosa [11] where *C. albicans* is known to enter the host. Therefore, DC-SIGN-positive DC might, through these C-type lectin receptors, form the first encounter with these pathogens and the host immune system and, after antigen presentation, initiate a cellular response [5;8].

It remains to be elucidated whether the destiny of the *C. albicans*-containing vesicles enriched in DC-SIGN is different from those enriched in MR. Though we observed some vesicles containing both receptors (not shown), it was intriguing that colocalization of both C-type lectins seemed not to occur in most *C. albicans*-containing vesicles. Therefore, it will be interesting to characterize these vesicles in more detail (this work is in progress in our laboratory) and to investigate if both lectins recognize similar or distinct carbohydrate moieties on *C. albicans*. This is of particular interest because MR is known as a recycling receptor, whereas DC-SIGN targets much deeper in the endosomal compartments [14].

Detailed knowledge of the recognition receptors for *C. albicans* on DC, together with the specific down-stream cellular events initiated by receptor engagement, may increase our understanding of possible immune dysfunctions in patients with mucocutaneous candidiasis and may offer new targets for immunotherapy of candidal infections and diseases.

Materials and Methods

Reagents and antibodies

FITC was from Fluka. Mannan and D-mannose were from SIGMA Chemical Co., St. Louis, MO. IL-4 and GM-CSF used for culturing monocyte-derived DC were from Schering-Plough (International, Kenilworth, USA). The following antibodies were used: AZN-D1, AZN-D3 (mouse IgG1, anti-DC-SIGN [11]); AZN-L50 (mouse IgG1, anti-ALCAM) [12] ; NKI-L19 (mouse IgG1 anti-$ß_2$ integrins); mAb clone 19.2 against MR was from BD Biosciences PharmIngen; the directly allophycocyanin-conjugated mAb against CD45RO was from Becton Dickinson; Cy5-conjugated goat-anti-mouse IgG was from Molecular Probes.

C. albicans culture conditions

C. albicans, strain UC820, a clinical isolate that has been well described [20], was maintained on agar slants at 4°C. Previous experiments showed that strain UC820 can develop hyphae and pseudohyphae *in vitro* and *in vivo* to the same extent as a panel of virulent control strains. *C. albicans* UC820 was inoculated into 100 ml of Sabouraud broth and was cultured for 24 h at 37°C. After three washes with pyrogen-free saline by centrifugation at 1500×g, the number of yeast cells was counted in a hemocytometer; occasional strings of two yeasts were counted as one colony-forming unit of *C. albicans*. The suspension was diluted to the appropriate concentration with pyrogen-free saline. Microscopy confirmed that the suspension consisted of blastoconidia. When necessary, the blastoconidia were heat-killed either at 56°C for 1 h or at 100°C for 30 min.

Cells

Immature DC were generated from human peripheral blood monocytes as described previously [9]. Briefly, monocytes were isolated by adherence to plastic and cultured in the presence of IL-4 (500 U/ml) and GM-CSF (800 U/ml) for 6-7 days. K562 transfectants either expressing DC-SIGN or ALCAM were generated by transfection of K562 cells with 10 µg of plasmid by electroporation as described previously [11;12]. Positive cells were sorted several times to obtain stable transfectants with expression levels of DC-SIGN and ALCAM similar to immature DC.

Immunofluorescence

Labeling of *Candida* cells was performed as follows: yeast cells were resuspended to 2 x 10^8/ml in 0.01 mg/ml FITC in 0.05 M carbonate-bicarbonate buffer (pH 9.5). After incubation for 15 min at room temperature in the dark, FITC-labeled *Candida* cells were washed twice in PBS containing 1% BSA (PBA buffer), heat-killed for 60 min at 56°C, and subsequently analyzed by flow cytometry.

DC were stained in PBA with primary antibodies and FITC-conjugated secondary antibodies and were analyzed by flow cytometry using the FACScalibur (BD Biosciences, Mountain View, CA). Isotype-matched controls were included.

C. albicans binding studies

DC or transfected K562 cells were stained with anti-CD45-allophycocyanin prior to exposure to FITC-labeled live or heat-inactivated *C. albicans* yeast forms. Before adding *C. albicans*, cells were or were not preincubated for 10 min at room temperature with mannose (100 mM), EGTA (5 mM), isotype control (mouse IgG1) or a mixture of anti-DC-SIGN mAb, AZN-D1 and AZN-D3 (20 µg/ml), in 20 mM Tris pH 8.0, containing 150 mM NaCl, 1 mM $CaCl_2$, 2 mM $MgCl_2$, and 1% BSA (TSA buffer) or, when EGTA was used, in PBS. Subsequently, FITC-labeled *C. albicans* were resuspended to the appropriate concentrations either in TSA or PBS and added in various cell:*Candida* ratios. After incubation, cell-*Candida* conjugates were analyzed by flow cytometry, and the relative difference in mean fluorescence intensity of the double-labeled events in comparison with that of control cells was calculated. Cells were labeled with anti-CD45-allophycocyanin to discriminate cells binding FITC-labeled yeast particles from yeast aggregates.

Fluorescent-bead adhesion assay

The fluorescent-bead adhesion assay was performed as described earlier [12;21]. Briefly, carboxylate-modified TransFluorSpheres (488/645 nm, 1.0 µm; Molecular Probes, Eugene, OR) were coated with ICAM-3-Fc or ALCAM-Fc, and adhesion was determined by measuring the percentage of cells that had bound fluorescent beads, by flow cytometry. In inhibition studies, the bead-adhesion assay was performed in presence of 0.3 mg/ml mannan, 5 mM EGTA, or 20 µg/ml antibodies against DC-SIGN or ALCAM.

Phagocytosis

Immature DC (5×10^5) were incubated with unopsonized heat-inactivated FITC-labeled *C. albicans* (2.5×10^6) in a total volume of 500 µl at 37°C in a water bath with orbital shaking at 150 rpm for 60 min. At the end of the incubation period, the DC binding *C. albicans* were separated from unbound *Candida* by a Ficoll gradient.

The samples were then mounted on poly-L-lysine-coated glass coverslips by centrifugation at 250 rpm for 3 min. Subsequently, the samples were fixed in 1% PFA in PBS for 15 min at room temperature, and permeabilized in cold methanol for 5 min on ice. After a blocking step in PBS/3% BSA for 60 min at room temperature, cells were labeled with monoclonal antibody (10 µg/ml in PBS/3% BSA) for 60 min at room temperature and subsequently incubated with Cy5-conjugated Goat-anti-Mouse $(Fab')_2$ fragments for 30 min at room temperature. Finally, samples were sealed in Mowiol and analyzed using a MRC1024 confocal microscope (Bio-Rad).

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Chapter 3

Human dendritic cells are less potent at killing *Candida albicans* than both monocytes and macrophages

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Abstract

Dendritic cells (DC) function as professional phagocytes to kill *Candida albicans* and subsequently present it to the adaptive immune system. Monocytes, macrophages and DC were generated from five individual donors and their *Candida*-killing capacity and cytokine release were assessed. Compared to monocytes and macrophages, DC from healthy volunteers were significantly less effective in *C. albicans*–stimulated cytokine release, killing of *C. albicans* blastoconidia and damaging of *C. albicans* hyphae. In conclusion, while important as antigen-presenting cells and initiators of the adaptive immune system, DC are poor in both intracellular killing and damaging of *C. albicans* hyphae. Effective handling of large numbers of *C. albicans* is the prime task of the innate immune system consisting of large numbers of neutrophils and monocytes.

Introduction

Candida albicans is present in the microflora of the digestive tract and mucocutaneous membranes of healthy individuals. While harmless under normal conditions, deficiencies in the host defense system or an imbalance in the normal microflora may lead to infections of the host with *C. albicans*. These infections can manifest either as acute or chronic candidiasis of the skin and mucosae, or as invasive or disseminated candidiasis, occurring primarily in the immunocompromized host. Mortality associated with disseminated candidiasis is around 30%, and has changed little despite the availability of new antifungal drugs [1, 2], while oropharyngeal and vulvovaginal candidiasis cause considerable morbidity in certain groups [3, 4].

In spite of the importance of *C. albicans* in human pathology, much has to be learned about the mechanisms through which this fungus is recognized by the immune cells to trigger the host defense. Neutrophils and macrophages are known to be the major cell populations involved in the host defense against candidal infection. It has been recently proposed that dendritic cells (DC) also play a central role in the defense against *C. albicans*. DC discriminate between the yeast and hyphal forms of *C. albicans* and initiate T-helper cell immunity, which is required for long-term protection against mucocutaneous candidiasis [5]. We have recently demonstrated that DC recognize and ingest *C. albicans* blastospores using the C-type lectin DC-SIGN [6]. It has been suggested that DC, for their function as antigen-presenting cells, not only phagocytose, process and present candidal antigens to T cells, but also kill *C. albicans* yeasts as efficient as macrophages do [7].

The aim of the present study was to extend these observations and compare the anticandidal properties of human DC, monocytes and macrophages in terms of proinflammatory cytokine release, intracellular killing of *C. albicans* blastospores, and extracellular damage of *C. albicans* hyphae.

Materials and Methods

Isolation of monocytes and generation of macrophages and DC

Blood mononuclear cells were isolated from five healthy volunteers as described elsewhere [8]. The adherent fraction of monocytes was obtained after 2 h incubation at 37°C and divided in three populations: one subset of monocytes was used for direct assessment of anti-candidal activities, one subset was allowed to differentiate into macrophages by incubation with 10% fresh human serum at 37°C for 7 days, and one subset was induced to transform into DC by incubation with recombinant IL-4 (500 U/ml) and granulocyte-macrophage colony-stimulating factor (800 U/ml, both from Schering-Plough, Kenilworth, USA) for 7 days [6]. The cell populations were phenotypically characterized through FACS analysis, by assessing the cell-membrane expression of CD14 and DC-SIGN, respectively **(Fig. 1)**.

Stimulation of cytokine production

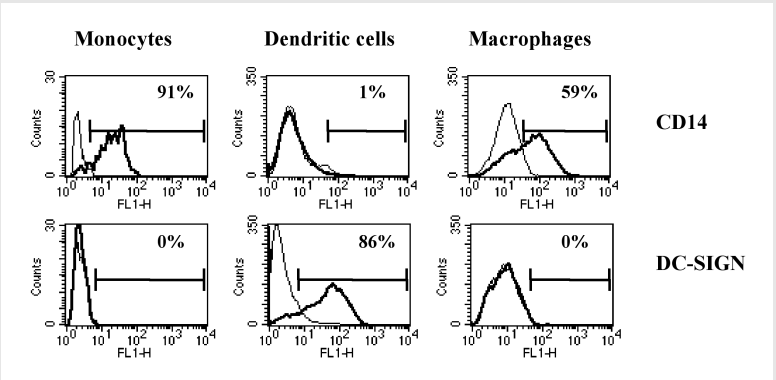
The monocytes, macrophages and DC were collected and resuspended in culture medium (RPMI 1640 supplemented with 10 mg/ml of gentamicin, 10 mM L-glutamine and 10 mM pyruvate), and the number was adjusted to $5x10^6$ cells/ml. Aliquots of $5x10^5$ cells in a 100 µl volume were incubated in 96-wells plates with 100 µl of either culture medium or heat-killed (30 min, 100°C) *C. albicans* yeasts or hyphae (ATCC 10231; 10^7 cfu/ml). Cytokine release was measured after 24 h of incubation at 37°C by specific commercial ELISA (BioSource International, Camarillo, CA; detection limit 16 pg/ml).

Candida-killing assays

Intracellular killing of *C. albicans* conidia was assessed microbiologically, as described [9]. Cells (5 x 10^5) in 100 µl of RPMI-dm were dispensed into the wells of a 96-well flat bottom plate (Costar) and incubated at 37°C and 5%

Figure 1

PHENOTYPIC CHARACTERIZATION OF MONOCYTES, MACROPHAGES AND DC. The various cell populations were characterized by measuring the expression of CD14 and DC-SIGN (CD209) by flow cytometry. Data are presented as an overlay graph of the isotype control (thin line) and the molecule of interest (bold line). Markers were set based on the results obtained with isotype controls (thin line). The percentage of positive cells, based on these markers is indicated.



CO_2. Cells were incubated with 1 x 10^4 cfu *C. albicans*, which were opsonised with 2.5% fresh mouse serum (E:T ratio, 40:1) in modified Eagle's medium (Gibco Life Technologies, Paisley, Scotland; MEM), for 45 min at 24°C. The choice of this particular cell-to-target ratio was carefully decided after initial pilot experiments, in such a way that the killing percentage in monocytes would be between 30% en 70%. This percentage in the control cell population is necessary in order to be able to demonstrate either increased or decreased killing of *Candida* in other cell populations (e.g. macrophages and DC).
After removal of the non-phagocytized *Candida* blastoconidia, 200 µl of culture medium, consisting of Sabouraud in MEM (50% v/v), was added to the monolayers. After 4 h of incubation at 37°C and 5% CO_2, the wells were gently scraped with a plastic paddle and washed with 200 µl distilled H_2O to achieve lysis of macrophages. This procedure was repeated three times, after which the pooled washes were adjusted to a final volume of 1 ml with distilled water. Microscopic examination of the culture plates showed that there was a complete removal of phagocytes. To quantify the number of viable intracellular *Candida* blastoconidia, 10-fold dilutions of each sample were spread on Sabouraud agar plates and incubated at 37°C for 24 h. The percentage of yeast killed by the macrophages was determined as follows: (1 – (cfu after incubation/ number of phagocytized cfu)) x 100. Cell-free incubations of blastoconidia were included as a control for yeast viability.
Extracellular hyphal damage was investigated by an XTT-conversion method, as previously described [9, 10]. *Candida* blastoconidia (strain UC820) grown on Sabouraud agar plates were suspended at a final concentration of 1 x10^6 cfu/ml in RPMI 1640 Dutch modification (with 20mM Hepes, without glutamine, ICN Biomedicals, Eschwege, Germany) supplemented with 1% gentamicin, 1% L-glutamine and 1% pyruvate. The pH of the suspension was adjusted to 6.4 using hydrochloric acid. Hyphae were obtained by incubating 10 ml of the suspension at 37°C for 24 h. After incubation, the hyphae were centrifuged (1800 rpm, 10 min) and resuspended in RPMI 1640 without phenol red and L-glutamine (RPMI-wp; ICN). Aliquots (160 µl) of a suspension containing 1 x 10^5 hyphae were dispensed into a 24-well flat bottom plate (Costar). Cells were collected and resuspended in RPMI: 8 x 10^5 cells (200 µl) were added to the wells containing hyphae at a final E:T ratio of 8:1, in the presence of 10% fresh mouse serum. Control wells contained either hyphae or cells only. After incubation for 2 h, 800 µl of sterile H_2O was added to the wells and the plate was rocked at room temperature to achieve lysis of cells. After 15 min., 800 µl sterile saline-solution containing XTT (2,3-bis-(2-methoxy-4-nitro-5-sulfophenyl)-2H-tetrazolium-5-carboxanilide; Sigma Chemical, St. Louis, MO) and coenzyme Q_0 (2,3-dimethoxy-5-methyl-1,4-benzoquinone; Sigma) were added to each well at a final concentration of 400 µg/ml XTT and 50 µg/ml coenzyme Q_0. After 1 h of incubation at 37°C, the plate was centrifuged (1800 rpm, 10 min) and 150 µl of the supernatant of each well was transferred to a well of a 96-well microtiter plate. The absorbance of each well was measured in a spectrophotometer at 450 nm. The percentage of fungal damage was defined as 1– ((A_{450} hyphae + PMN – A_{450} PMN) / E_{450} hyphae) x 100.

Statistical analysis

Five separate experiments were performed in duplicate on separate days, and the results presented are pooled data. The comparison between the various cell populations isolated from the same healthy donors were analysed by paired non-parametric Wilcoxon test. The level of significance was set at 0.05.

Results

When the different cell populations from the same volunteers were compared, DC were significantly less effective in the intracellular killing of *C. albicans* blastoconidia (13.6 ± SD 5.4% after 3 h of incubation), than macrophages or monocytes (25.7 ± 12.3% and 34.7 ± 10.7%, respectively, $p<0.05$; **Fig. 2**). Similarly, hyphal damage induced by DC was lower (29.8 ± 12.7% after 3 h of incubation) than that induced by macrophages (61.8 ± 16.7%) or monocytes (72.7 ± 12.1%, $p<0.05$; **Fig. 2**).

Although DC produced significant amounts of tumor necrosis factor (TNF, 243 ± 34 and 370 ± 65 pg/ml) and interleukin-8 (IL-8, 2430 ± 840 and 12910 ± 2345 pg/ml) after stimulation with *C. albicans* blastoconidia or hyphae, TNF and IL-8 release by DC was only 5-10% of that released by monocytes or macrophages (**Fig. 3**, $p<0.01$). Virtually no release of IL-6 by DC was measured, whereas both monocytes and macrophages produced significant amounts of these cytokines. The interferon-γ (IFN-γ) production stimulated by conidia was also lower after stimulation of DC, whereas its release after stimulation with candidal hyphae was similar between the three cell populations **(Fig. 3)**.

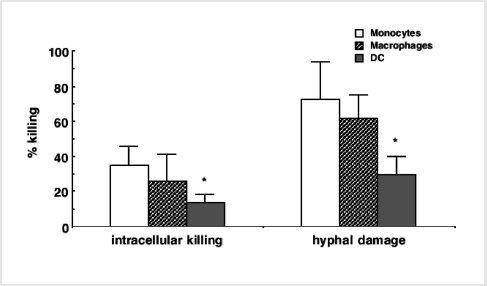


Figure 2

KILLING OF *C. ALBICANS* CELLS BY MONOCYTES, MACROPHAGES AND DC. Intracellular killing of yeasts or extracellular damage to hyphae of *C. albicans* by monocytes (open bars), macrophages (hatched bars) or DC (solid bars) was assessed after 3 h of incubation at 37°C. Data are presented as means + SD of percentages of killing (n=5, *p<0.05 by Mann-Whitney U-test).

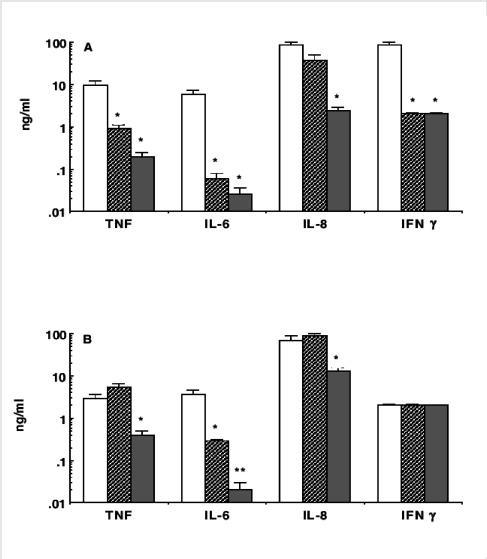


Figure 3

STIMULATION OF PROINFLAMMATORY CYTOKINE RELEASE FROM HUMAN MONOCYTES, MACROPHAGES AND DC BY *C. ALBICANS*. Monocytes (open bars), macrophages (hatched bars) or DC (solid bars) were stimulated with 10^7 cfu/ml heat-killed *C. albicans* yeasts (panel A) or hyphae (panel B). TNF, IL-6, IL-8 and IFN-γ were measured 24 h later. Cytokine production in unstimulated cells was below the detection limit. Data represent means + SD of 5 volunteers (*p<0.05, **p<0.01; by Mann-Whitney U-test).

Discussion

Recent studies have suggested that DC have a role as professional candidacidal cells, and that they are as efficient as macrophages in killing *C. albicans* blastoconidia [7]. In the present study, we have extended these observations by comparing DC with monocytes and macrophages from the same healthy volunteers. In contrast to the previous report of Newman and Holly [7], we found that DC are significantly less efficient than monocytes and macrophages in both intracellular killing of blastoconidia and extracellular hyphal damage.

The differences between our data and the previous reports are likely due to differences in the methodology. We have recently shown that assays using yeast-phagocyte suspensions may be inappropriate for the assessment of intracellular killing of *C. albicans* yeasts, due to strong adherence of *C. albicans* to the test tubes, or clumping of the yeast cells [9]. These methods may result in artifacts suggesting increased candidacidal activity. It cannot be excluded that such mechanisms may have played a role in earlier reports, reporting 80-90% killing of *C. albicans* blastoconidia by DC, while agents inhibiting either phagocytosis or oxygen radical and nitric oxide production failed to decrease the observed killing [7].

It has been demonstrated earlier that *C. albicans* is phagocytosed and processed by DC for presentation of candidal antigen [5, 7], and pulsing of DC with fungal RNA induces protective immunity to *C. albicans* infection [11]. It has therefore been assumed that DC kill *C. albicans* blastoconidia during the process of phagocytosis and presentation. Our data confirm this assumption but demonstrate, however, that DC are not as efficient as monocytes and macrophages are in killing *C. albicans* cells. This is in agreement with the concept that the function of DC is to present candidal antigens to T cells [12], rather than eliminating the microorganism, whereas neutrophils and macrophages are the main populations involved in elimination of the microorganisms [13-15]. Taking into account the low numbers of DC present at the site of infection, compared to the numbers of neutrophils and macrophages, DC are unlikely to play a significant role in the elimination of *C. albicans* organisms at the site of infection.

In addition to antigen presentation, DC are known to be able to produce proinflammatory cytokines after stimulation with *C. albicans* cells [5], and we have confirmed this observation by showing a significant release of TNF and IL-8 after challenge with *C. albicans* blastoconidia and hyphae. However, the cytokine production by DC was much lower than that by monocytes and macrophages, and virtually no release of IL-6 was found. It is likely that the low amounts of proinflammatory cytokines exert paracrine and autocrine effects during antigen presentation, rather than functioning as potent activators of anticandidal effector cells such as neutrophils and macrophages. Interestingly, differences in the cytokine production between monocytes and macrophages were observed. Whereas monocytes released greater amounts of cytokines upon stimulation with blastoconidia compared to hyphae, the opposite was found for macrophages, which produced more TNF and IL-8 upon stimulation with hyphal forms. This suggests an adaptive response during the phenotypic switch of *C. albicans* from the yeast form to the hyphal form of the fungus. The yeast form of *Candida* is most of the time the phenotypic form which invades the bloodstream and encounter monocytes, whereas hyphae cause invasion of the tissues, where they are recognized by tissue

macrophages. As the phenotypic switch is considered an important virulence trait of *C. albicans* species, the monocyte/macrophage cell populations seem to be adapted to encountering a specific phenotypic form of the fungus.
In conclusion, although DC are capable of phagocytosing and processing *C. albicans* blastoconidia for antigen presentation and initiating the adaptive immune response [16], they only release proinflammatory cytokines and kill *C. albicans* cells to a limited extent, when compared with monocytes and macrophages. Those innate immune responses provide control of the pathogen early on, until the adaptive immune response is launched.

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Chapter 4

Relevance of DC-SIGN in DC-induced T cell proliferation

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Abstract

The role of dendritic cell-specific ICAM-3 grabbing non-integrin (DC-SIGN) in DC-T cell communication was assessed by analyzing the effect of DC-SIGN blocking mAbs in MLR. The results show that the degree of inhibition by DC-SIGN and LFA-1 mAbs depends on the magnitude of the MLR and the maturation status of the DC. Addition of DC-SIGN mAbs at several time points during MLR showed that DC-SIGN is involved early on in DC-T cell contacts. This initial role is masked by strong adhesive and costimulatory mechanisms, indicating a short-lived effect of DC-SIGN in DC-T cell interactions.
To examine this concept in more detail, the percentage of PBL capable of binding DC-SIGN was determined. Analysis of several donors revealed that 1 to 20% of PBL bind to beads coated with recombinant DC-SIGN, and the DC-SIGN-binding cells comprised all major cell subsets found in blood. PBL isolated from a donor with high DC-SIGN-binding capacity were more prone to blocking by DC-SIGN mAbs in MLR than PBL from a donor with low DC-SIGN binding capacity. This study indicates an initial and transient role for DC-SIGN in T cell proliferation, which becomes apparent when T cell proliferation is low and when the percentage of DC-SIGN binding PBL is high.

Introduction

Dendritic cells (DC) and T cells are important players of the immune system. Interactions between these two cell types can result in the induction of antigen-specific immunity or in tolerance [1]. DC are professional APC and reside in peripheral tissues on the alert for invading pathogens. After encountering and processing antigen, the DC will migrate to the secondary lymphoid organs to present peptides in MHC context to T cells [2]. During inflammation DC mature and acquire properties to induce an effective immune response. In non-inflamed situations however, DC remain immature and induce tolerance [1,2]. In the lymph nodes, naïve T cells and DC interact transiently in an antigen-independent manner to enable the T cells to inspect a large number of MHC molecules on DC for the presence of a specific peptide [3]. ICAM-3 is an important adhesion molecule in these antigen-independent interactions between T cells and APC [4]. Binding partners for ICAM-3 include the β2-integrins Leukocyte Functional Antigen-1 (LFA-1) [5] and αdβ2 [6], and the C-type II lectin DC-specific ICAM-3 grabbing non-integrin (DC-SIGN) [7]. This C-type II lectin is expressed *in vitro* on monocyte-derived DC and *in vivo* on DC in several tissues including lymph nodes, cervix, mucosa and skin [7,8]. In addition, DC-SIGN expression is detected on a subset of myeloid blood DC and on blood DC antigen 2^+ plasmacytoid DC precursors [8,9]. As integrins need activation before appropriate binding can occur, DC-SIGN was put forward as an initial binding partner of ICAM-3 [7]. Upon specific peptide recognition by a T cell, the transient adhesive interactions between T cells and DC are strengthened, resulting in an immunological synapse [3]. This structure is formed by the interactions of several adhesion and costimulatory molecules such as LFA-1 interacting with ICAM-1 and/or ICAM-3 and CD28 interacting with CD80 and/or CD86 and provides a platform for sustained TCR engagement and signaling [10-12]. Such a mechanism predicts a transient role for DC-SIGN in DC-T cell interactions and may explain in part the conflicting data obtained in T cell activation studies so far [7,13-16]. Studies of Geijtenbeek *et al.* [7] and Puig-Kroger *et al.*[13] showed that blocking DC-SIGN mAbs have an inhibitory effect on the proliferation of resting T cells induced by allogeneic DC or THP-1 cells differentiated into DC. In contrast, Granelli-Piperno *et al.* [16] did not show a requirement of DC-SIGN in MLR driven by DC. Real *et al.* [14] could not confirm a role for DC-SIGN in early DC-T cell contact, as their studies did not reveal an effect of blocking DC-SIGN on immature DC on $CD4^+$ T cell on CD69 expression, motility, and Ca^{2+} response. However, Martinez *et al.* [15] analyzed early T cell activation as well by measuring IL-2 and IFN-γ secretion by $CD4^+$ T cells and showed that DC-SIGN can modulate this secretion dependent on the strength of T the cell stimulus. Moreover, these authors showed a modest down-regulation of CD69 expression on $CD4^+$ T cells in the presence of Chinese hamster ovary cells transfected with DC-SIGN.

In the present study, numerous MLR, driven by immature or mature DC, were performed to unravel the relevance of DC-SIGN in MLR. We observed that blocking DC-SIGN only affects T cell proliferation when immature DC are used and when the proliferative response is weak. Likewise, inhibition of T cell proliferation by LFA-1 blocking mAbs is also dependent on the strength of the T cell response. Moreover, the effect of blocking DC-SIGN in MLR depends on the percentage of PBL that are able to bind DC-SIGN.

Materials and Methods

Cells

Immature DC were generated from human PBMC as described previously [7]. Briefly, PBMC were isolated from buffy coats of donated blood obtained from healthy individuals by Ficoll density centrifugation. Monocytes were isolated from PBMC by adherence to plastic and cultured in the presence of IL-4 and GM-CSF (500 and 800 U/ml, respectively; Schering-Plough, Brussels, Belgium) in RPMI 1640 medium (Gibco, Invitrogen, Breda, The Netherlands) containing 10% FCS (Greiner Bio-One B.V. Alphen aan den Rijn, The Netherlands) for 6 days. Non-adherent cells (PBL) were collected for later use. Mature DC were generated from immature DC by adding 2 μg/ml LPS (Sigma, Sigma-Aldrich Chemie B.V. Zwijndrecht, the Netherlands) at day 6. Mature DC were harvested on day 7.

For testing DC-SIGN expression, DC were labelled in PBS containing 0.5% BSA and 0.01% sodium azide with mAb against DC-SIGN (5 μg/ml AZN-D1) or with isotype control mouse IgG1 (5 μg/ml, BD Biosciences, San Diego, CA, USA) for 30 min at 4°C. After a washing step, DC were labelled with FITC-conjugated goat-anti-mouse IgG1 for 30 min at 4°C. After a final washing step, the labelled DC were analyzed by flow cytometry (FACScalibur, BD Biosciences, Mountain View, CA, USA).

HSB-2 cells were cultured in IMDM (Gibco, Invitrogen, Breda, The Netherlands) supplemented with 5% FCS.

T cell proliferation assays

T cell proliferation was assessed by [^{3}H]-thymidine incorporation or by CFSE methodology after 6 or 7 days, respectively. For the CFSE methodology; the PBL, resuspended at a density of 2×10^7 cells per ml in PBS, were labeled with 5 μM CFSE (Molecular Probes, Leiden, The Netherlands) for 10 min at room temperature (RT). Free CFSE was quenched by the addition of an equal volume of FCS. The labeled cells were washed twice in PBS and resuspended in RPMI 1640 medium containing 10% FCS [17]. PBL comprise mainly T cells, B cells, and NK cells. As those T and B cells are from the same donor no MLR will be induced by DC-SIGN expressed by B cells [18]. MLR was induced by allogeneic DC. Unlabeled or CFSE-labeled PBL (1×10^5) were cocultured with 1.5×10^3 allogeneic DC in 96-wells round bottom plates for 6-7 days. At 0, 24, and 72 h after the start of the experiment, blocking mAbs directed against DC-SIGN (10 μg/ml AZN-D1 and 10 μg/ml AZN-D3), LFA-1 (10 μg/ml NKI-L15), or CD6 (5 μg/ml, clone M-T605, mouse IgG1, BD Pharmingen) were added where indicated. Control mouse IgG1 (10 μg/ml, R&D Systems, Abingdon, UK) or total mouse IgG (10 μg/ml, Jackson ImmunoResearch, West Grove, PA, USA) were included as controls. After 6-7 days, PBL proliferation was assessed by determining CFSE staining intensity by flow cytometry (FACScalibur, BD Biosciences, Mountain View, CA) or by measuring [^{3}H]-thymidine incorporation (1 μCi/well, 8-16 h pulse; MP Biomedicals Inc., Irvine, CA, USA). To enable comparison of data obtained using different donors, proliferation data obtained under control conditions were expressed as 100% proliferation, and putative blocking was calculated relative to the control MLR for that given donor.

Conjugate formation assay

Allogeneic DC (10^5 cells) were mixed with 10^6 PBL and incubated for 15 min at 37°C while shaking. The DC-PBL mixture was allowed to adhere on poly-L-lysine (50 µg/ml)-coated glass slides for 5 min at RT. Next, cells were fixed with 2% paraformaldehyde in PBS at RT for 20 min. Non-specific binding sites were blocked with blocking buffer (3% BSA, 10 mM glycine and 1% human serum in PBS) at RT for 1 h. Cells were triple stained with anti-DC-SIGN (DCN46, 10 µg/ml), anti-ICAM-3 (AZN-IC3, 10 µg/ml), and anti-CD3 (T3B, 10 µg/ml) in blocking buffer at RT for 1 h, followed by incubation with isotype-specific Alexa-488, -568, or –647-conjugated goat-anti-mouse mAbs for 1 h. Samples were analyzed using a MRC1024 confocal microscope (Bio-Rad, Hercules, CA, USA) with a 60× objective.

DC-SIGN-His construct

Recombinant DC-SIGN-His consists of the extracellular domain of DC-SIGN harboring a six-His-tag at the N-terminus and was kindly provided by Dr. Clark (University of Washington, Seattle) [19]. Bacterial strain *Escherichia coli* M15(Prep4) was transfected with this construct, and expression was induced by 0.1 mM isopropylthiogalactoside (Sigma-Aldrich Chemie B.V. Zwijndrecht). The protein was solubilized in 8.5 M Urea and subsequently refolded by stepwise dialysis against buffers containing decreasing urea concentrations. The protein preparation was incubated with mannan agarose beads (Sigma-Aldrich Chemie B.V. Zwijndrecht). Only functional protein is able to bind to mannan agarose beads. After several washing steps to remove unbound protein, the functional DC-SIGN was eluted from the beads by EGTA, removing the essential Ca^{2+}-ion from DC-SIGN. After removal of the beads by centrifugation, the supernatant containing the functional protein was reconstituted with Ca^{2+}-ion containing buffer (20 mM Tris-HCl, pH 8.0, 150 mM NaCl, 1 mM $CaCl_2$, 2 mM $MgCl_2$ and 0.5% BSA). The six-His tagged, humanized, single chain h5G1.1 antibody was used as control and kindly provided by Dr. Kretz-Rommel [20].

Binding assay with soluble DC-SIGN-His construct

The soluble DC-SIGN–His construct (10 µg/ml) was incubated with 5×10^4 PBL in binding buffer (20 mM Tris-HCl, pH 8.0, 150 mM NaCl, 1 mM $CaCl_2$, 2 mM $MgCl_2$ and 0.5% BSA) in a 96-well V-shaped bottom plate at 37°C for 30 min. Where indicated, DC-SIGN-His constructs were preincubated with 100 µg/ml mannan (from Saccharomyces cerevisiae, Sigma-Aldrich Chemie B.V. Zwijndrecht) prior to the binding assay at RT for 20 min. Subsequently, the samples were incubated with mouse-anti-Penta-His Alexa-488 (Qiagen, Benelux B.V., Venlo, The Netherlands) at 37°C for 30 min. Cells were analyzed by flow cytometry and the percentage of cells that had bound recombinant DC-SIGN-His was quantified.

Bead-binding assay with DC-SIGN–His construct

DC-SIGN–His or control-His were coated onto streptavidin-coated, carboxylate-modified TransFluoSpheres (488/645 nm, 1.0 µm; Molecular Probes [21]). First, streptavidin-coated beads were incubated with biotinylated horse-anti-mouse IgG (10 µg; Vector, Brunschwig Chemie, Amsterdam, the Netherlands) at 37°C for 2 h, followed by an overnight incubation with mouse-anti-Penta-

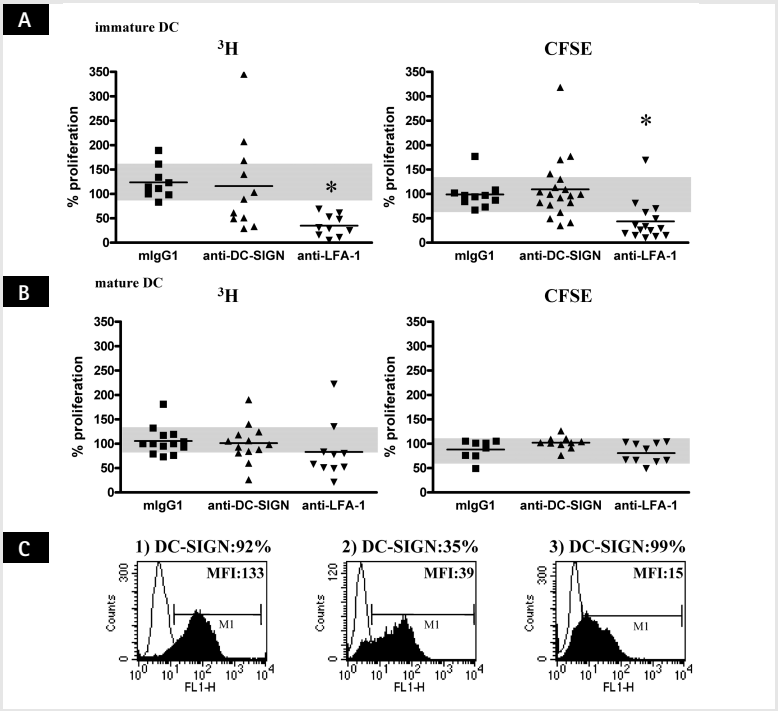
His (1 µg; Qiagen) at 4°C. Subsequently, the beads were incubated with 250 ng DC-SIGN-His or control-His at 4°C for two days.
The DC-SIGN and control beads were incubated with 5×10^4 PBL or HSB-2 cells in binding buffer (20 mM Tris-HCl, pH 8.0, 150 mM NaCl, 1 mM $CaCl_2$, 2 mM $MgCl_2$ and 0.5% BSA) in a 96-well V-shaped bottom plate at 37°C for 30 min. Where indicated, the beads were preincubated with 100 µg/ml mannan, 5 mM EGTA, or 100 µg/ml soluble ICAM-3 (Fc-ICAM-3 [7]) prior to the binding assay at RT for 20 min. In some experiments, PBL or HSB-2 cells were pre-incubated with 40 µg/ml of ICAM-3 blocking mAbs for 20 min at RT prior to the binding assay (CBR3/1 and 3/2 [7]). After washing, adhesion of the beads to the cells was assessed by flow cytometry.

Characterization of DC-SIGN-binding cells by flow cytometry

To characterize the DC-SIGN binding cells, PBL were incubated with DC-SIGN-His coated beads as described above. After washing, the cells were labeled for 20 min at RT with combinations of CD3-FITC (DakoCytomation B.V., Heverlee, Belgium), CD3-PE (BD Pharmingen), CD4-PE (BD Pharmingen), CD8-FITC (BD Pharmingen), CD19-PE (BD Pharmingen), CD25-PE (Becton Dickinson), CD45RA-PE (Immunotech), CD45RO-FITC (DakoCytomation B.V.), CD56-PE (IQ Products, Groningen, the Netherlands) and/or CD62L-FITC (DakoCytomation B.V.). Samples were analyzed by flow cytometry (Cytomics Fc 500, Beckman Coulter, Fullerton, CA). The percentage cells, which had simultaneously bound beads and the mAb under study, was measured.

Figure 1

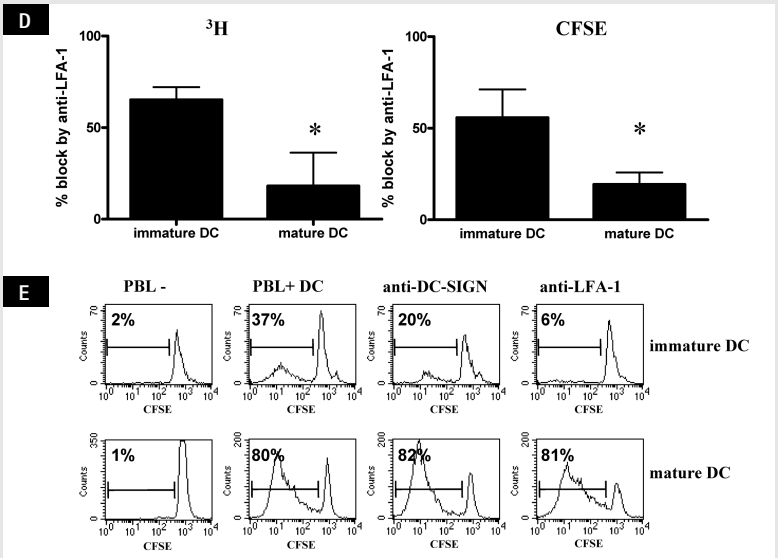
DC-MATURATION DEPENDENT EFFECTS OF DC-SIGN AND LFA-1 ON T CELL PROLIFERATION. PBL ($1x10^5$) were cocultured with $1.5x10^3$ immature (A) or mature (B) allogeneic DC. After 6-7 days, proliferation was assessed by [^{3}H]-thymidine incorporation or by CFSE dilution. Control mouse IgG1 or blocking mAbs (10 μg/ml) directed against DC-SIGN (AZN-D1 and AZN-D3) or LFA-1 (NKI-L15) were added at the onset of the experiment. Data are percentages of control (no addition of mAbs). Each dot represents the average of one independent experiment performed in triplicate. Each MLR experiment consists of a different allogeneic DC-PBL combination. Significant difference from mouse IgG control as determined by ANOVA, followed by the Student Newman Keuls test: * $p<0.05$. Confidence interval (99%) of control mouse IgG1,



Results

DC maturation state affects contribution of DC-SIGN and LFA-1 in MLR

The role of DC-SIGN in T cell proliferation is controversial [7,13-16]. To unravel the significance of DC-SIGN in T cell proliferation, numerous MLR were performed in the presence of DC-SIGN-blocking mAbs, and mAb directed against LFA-1 was included as a positive control for blocking the MLR. Immature and mature allogeneic DC were used as T cell stimulators. T cell proliferation was determined by [^{3}H]-thymidine incorporation or by CFSE dilution **(Fig. 1A, B)** which yielded comparable results. As expected, with immature DC, a consistent block in T cell proliferation is observed when LFA-1 mAb is present **(Fig. 1A).** Using both methodologies, significant blocking was observed for LFA-1, as determined by ANOVA, followed by the Student Newman Keuls test: * $p<0.05$. Taking the group as a whole, no significant effect of



assessed by [^{3}H]-thymidine incorporation (immature DC; 86-161%, mature DC; 81-129%) and CFSE dilution (immature DC; 67-130%, mature DC; 63-112%), represented by gray area. (C) DC-SIGN (AZN-D1) expression on immature DC from three different donors. These DC were cocultured with different allogeneic PBL donors. Proliferation was assessed after 7 days (CFSE dilution). The percentage of proliferation with DC-SIGN mAb (relative to control) is depicted for each donor. Transparent histograms represent mIgG1 isotype control, and filled histograms represent DC-SIGN mAb. MFI, mean fluorescence intensity. (D) Percentage of block in T cell proliferation mediated by LFA-1 mAb in MLR driven by allogeneic immature and mature DC. Data expressed as percentages of control (no addition of mAbs), are mean ± SEM of 10 independent experiments, assessed by [^{3}H]-thymidine incorporation or by CFSE dilution. Each MLR experiment consists of a different allogeneic DC-PBL combination. Significant difference from immature DC, as determined by unpaired two-tailed Student's t-test: * $p<0.05$. (E) CFSE profiles of T cells stimulated with immature DC or mature DC in the presence and absence of blocking mAbs. Proliferation was assessed after 7 days. In this experiment, the same allogeneic DC-PBL combination was used.

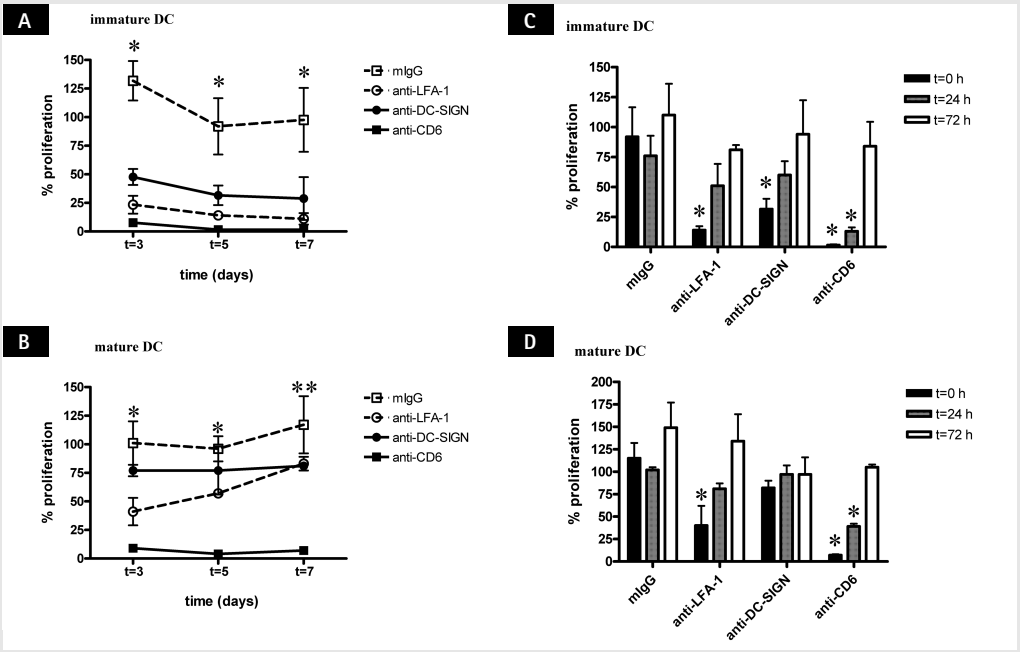
blocking DC-SIGN mAbs was observed in agreement with others [16]. However, in a subgroup of DC-T cell donor pairs, the presence of DC-SIGN mAbs resulted in less T cell proliferation, as illustrated by their location outside the 99% confidence interval of control mouse IgG1. No correlation between expression profile of DC-SIGN on immature DC and the extent of the DC-SIGN effect on the T cell proliferation was observed **(Fig. 1C)**.

When mature DC were used as stimulator cells **(Fig. 1B)**, hardly any blocking effect is observed with DC-SIGN mAbs. It is surprising that as LFA-1 is an effective mediator in MLR [22,23], the blocking potential of the LFA-1 mAb is also decreased considerably when mature DC are used as T cell stimulators **(Fig. 1B+D)**. The blocking effect of DC-SIGN mAbs is observed when using immature DC as stimulator cells, and the blocking effect is absent when mature DC are used as stimulator cells **(Fig. 1E)** using the same donor for raising immature as well as mature DC and the same allogeneic PBL donor.

Together, these data show that the use of mature DC as T cell stimulators abrogates the contribution of DC-SIGN and LFA-1 in MLR.

Figure 2

TIME-DEPENDENT, DIFFERENTIAL EFFECTS OF DC-SIGN, LFA-1, AND CD6 ON T CELL PROLIFERATION. (A, B) PBL ($1x10^5$) were cocultured with $1.5x10^3$ immature (A) or mature (B) allogeneic DC. After 3, 5 and 7 days, proliferation was assessed by [^{3}H]-thymidine incorporation. Blocking mAbs (5-10 μg/ml) against LFA-1 (NKI-L15), DC-SIGN (AZN-D1 and AZN-D3) and CD6 or control mouse IgG were added at the onset of the experiment. Results are expressed as the mean percentage of the control (no addition of mAbs) from triplicate wells. One representative experiment out of three is shown. (A) *Time (t) =3, 5, and 7; significant difference from anti-DC-SIGN, anti-LFA-1, and anti-CD6 as determined by ANOVA, followed by the Student Newman Keuls test: $p<0.01$. (B) *t=3, 5; significant difference from anti-LFA-1 and anti-CD6, as determined by ANOVA, followed by the Student Newman Keuls test: $p<0.05$. **T=7; significant difference from anti-DC-SIGN, anti-LFA-1 and anti-CD6, as determined by ANOVA, followed by the Student Newman Keuls test: $p<0.05$. (C, D) PBL ($1x10^5$) were cocultured with $1.5x10^3$ immature (C) or mature (D) allogeneic DC. Blocking mAbs (5-10 μg/ml) against LFA-1 (NKI-L15), DC-SIGN (AZN-D1 and AZN-D3) and CD6 or control mouse IgG were added to the cocultures at the onset of the experiment (t=0), after 24 h, or after 72 h. Proliferation was assessed by [^{3}H]-thymidine incorporation after 6 days. Results, expressed as percentage of the control (no addition of mAbs), are the mean ± SD of triplicate wells. A representative experiment out of three is shown. Significant difference from control mouse IgG according to ANOVA, followed by the Student Newman Keuls test: * p<0.01. In (A) and (C) the same allogeneic, immature DC-PBL combination was used, that could be blocked by DC-SIGN mAbs. (B and D) The same allogeneic, mature DC-PBL combination was used.



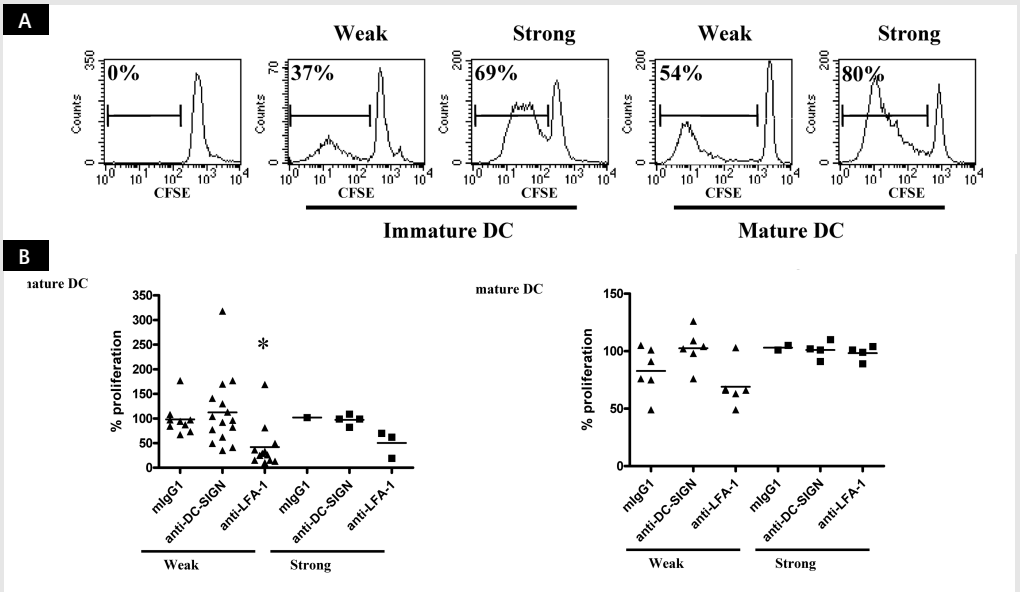
DC-SIGN, LFA-1, and CD6 differentially influence DC-induced T cell proliferation over time

A full-blown T cell response, when measured at day 7, results in a waning effect of DC-SIGN and LFA-1 mAbs in MLR driven by mature DC. This could also hold for DC-SIGN in MLR driven by immature DC. Therefore, T cell proliferation was assessed after 3, 5 and 7 days. As positive control for blocking MLR driven by mature DC, a CD6 blocking mAb was included, as CD6 plays a crucial role in mature DC-T cell interactions [24]. In immature DC-T cell donor pairs, which could be blocked by DC-SIGN mAbs, as depicted in **Figure 1A**, LFA-1, DC-SIGN and CD6 mAbs blocked T cell proliferation at all time points indicated **(Fig. 2A)**. However, T cell proliferation in DC-T cell donor

pairs, which could not be blocked by DC-SIGN mAbs when measured after 7 days, also did not show a blocking effect of DC-SIGN mAbs when measured after 3 and 5 days (data not shown). It is remarkable in contrast to LFA-1 and DC-SIGN mAbs that only the CD6 mAb was able to block proliferation induced by mature DC at all time points indicated **(Fig. 2B)**. LFA-1 mAb effectively blocked T cell proliferation induced by mature DC when measured after 3 days, but after 7 days the blocking effect was hardly detectable. To further characterize the effect of blocking DC-SIGN during MLR, blocking mAbs against DC-SIGN were added at 0, 24 or 72 hours after onset of the immature or mature DC-T cell coculture **(Fig. 2C, D)**. The effects of blocking mAbs to LFA-1 and CD6 were evaluated in parallel. In immature DC-T cell donor pairs, which could be blocked by DC-SIGN mAbs, as depicted in **Figure 1A**, LFA-1 and DC-SIGN mAbs blocked T cell proliferation effectively, only when added at the onset of the DC-T cell coculture **(Fig. 2C)**. Similarly, LFA-1 mAb inhibited T cell proliferation induced by mature DC, only when added at the onset of the experiment **(Fig. 2D)**. In contrast, CD6 mAb effectively blocked T cell proliferation induced by both immature and mature DC when added at 0 and 24 hours after onset of the experiment.
Altogether, these kinetic blocking experiments show that DC-SIGN and LFA-1 play a role early on in MLR whereas CD6 mediates a long-lived effect.

Figure 3

DC-SIGN AND LFA-1 MABS MAINLY INHIBIT LOW RESPONDER MLR. CFSE-labeled PBL ($1x10^5$) were cocultured with $1.5x10^3$ immature or mature allogeneic DC. After 7 days, proliferation was determined by flow cytometry. (A) CFSE histogram profiles of allogeneic PBL stimulated with immature DC or mature DC. PBL and DC were used from four different PBL and DC donors. Markers define the percentage of proliferating cells. One representative experiment out of five is shown. (B, C) T cell proliferation in MLR with immature (B) and mature DC (C) in the presence of DC-SIGN (AZN-D1 and AZN-D3) or LFA-1 (NKI-L15)-blocking mAbs added at the onset of the experiment. MLR were separated into weak (<60% of PBL proliferate) and strong (>60% of PBL proliferate) MLR responses. Data are percentages of control (no addition of mAbs). Each dot represents the average of one independent experiment performed in triplicate. Each MLR experiment consists of a different allogeneic DC-PBL combination. Significant difference from control mouse IgG according to ANOVA, followed by the Student Newman Keuls test: * $p<0.05$.



Contribution of DC-SIGN and LFA-1 to MLR depends on strength of T cell response

In MLR, several T cells will be activated upon recognition of specific MHC peptide complexes expressed by the allogeneic DC. The number of T cells reacting to these MHC peptide complexes varies between different donors and consequently, the strength of the MLR response will vary. Indeed, in some MLR, a very strong T cell-proliferative response was detected, whereas in others, the response was rather weak **(Fig. 3A)**. This prompted us to separate the MLR into weak and strong responses based on the percentage of proliferating T cells as determined by CFSE staining. Based on several experiments, MLR, in which after 7 days, less than 60% of the T cells were detected in the proliferative pool could be defined as a weak MLR, whereas MLR, in which more than 60% of the T cells were detected in the proliferative pool, could be defined as a strong MLR. When taking the group as a whole, no blocking effect of anti-DC-SIGN mAbs was observed. However, when categorized in this way, the effects of blocking DC-SIGN mAbs were only visible in several weak MLR and when immature DC were used as stimulators **(Fig. 3B)**. Similar to DC-SIGN mAbs, LFA-1 mAb mostly affected MLR driven by immature DC, and the blocking effect was strongest in the weak MLR **(Fig. 3C)**.
Thus, in strong MLR, proliferation cannot be blocked by DC-SIGN mAbs and are blocked less efficiently by LFA-1 mAb.

Localization of DC-SIGN in DC-T cell contact area

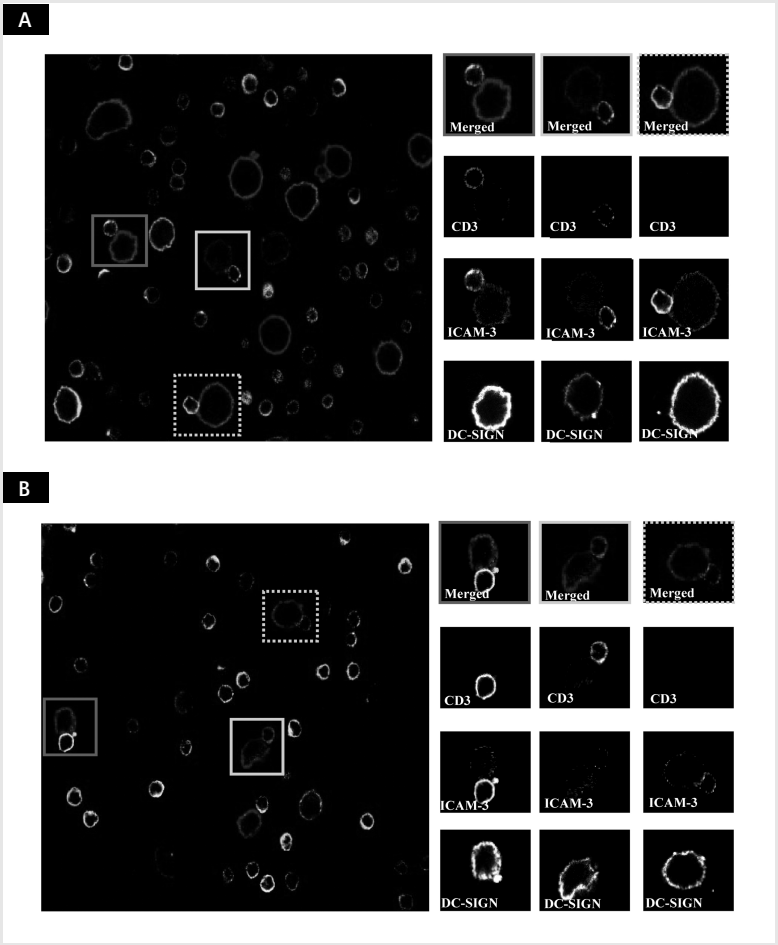
To visualize the behavior of DC-SIGN in the DC-T cell contact area, we performed DC-T cell conjugate formation experiments. Immature and mature DC formed contacts with T cells **(Fig. 4)**. DC-SIGN is present in the DC-T cell contact area, although DC-SIGN is not recruited to the contact zone. No difference in distribution of DC-SIGN was observed between immature **(Fig. 4A)** and mature **(Fig. 4B)** DC-T cell conjugates. Participation of $CD3^-$ cells in the DC-PBL conjugates or enrichment of ICAM-3 in the contact site did not influence the localization of DC-SIGN.
In conclusion, these data show that DC-SIGN is not enriched in the DC-T cell contact area.

Characterization of DC-SIGN binding to PBL

Various studies indicate that DC-SIGN-ICAM-3 interactions are of a transient nature [7,25]. To examine this in more detail, we analyzed the binding capacity of PBL derived from various donors to a DC-SIGN-His construct used in free form or attached to streptavidin-coated fluorescent beads. DC-SIGN-His-coated beads showed the highest binding to PBL as compared with soluble DC-SIGN-His, with an average of 5.4% specific binding **(Fig. 5A)**. It is interesting that PBL of some donors showed a much higher binding to DC-SIGN-His coated beads. DC-SIGN-specific binding was calculated by subtracting binding data determined in the presence of mannan. Determination of specific binding with EGTA and control-His beads was comparable with binding in the presence of mannan **(Fig. 5B)**. PBL isolated from a donor whose PBL have a high DC-SIGN-binding capacity (22%) were labeled with subset-specific mAbs to characterize the cells that bind DC-SIGN **(Fig. 5C)**. All cell subsets displayed a distinct percentage of cells that were positive for DC-SIGN binding, which was highest for B cells **(Table 1)**. Of the B cells

Figure 4

PRESENCE OF DC-SIGN IN DC-T CELL CONTACT AREA. Overview and zoomed-in pictures of conjugates between PBL and immature (A) or mature (B) DC. Allogeneic DC and PBL were incubated at 37°C for 15 min and subsequently mounted on poly-L-lysine coated coverslips. Cell conjugates were stained for DC-SIGN (blue), ICAM-3 (green) and CD3 (red). Results represent two different allogeneic DC-PBL combinations. Representative pictures of three independent experiments are shown.

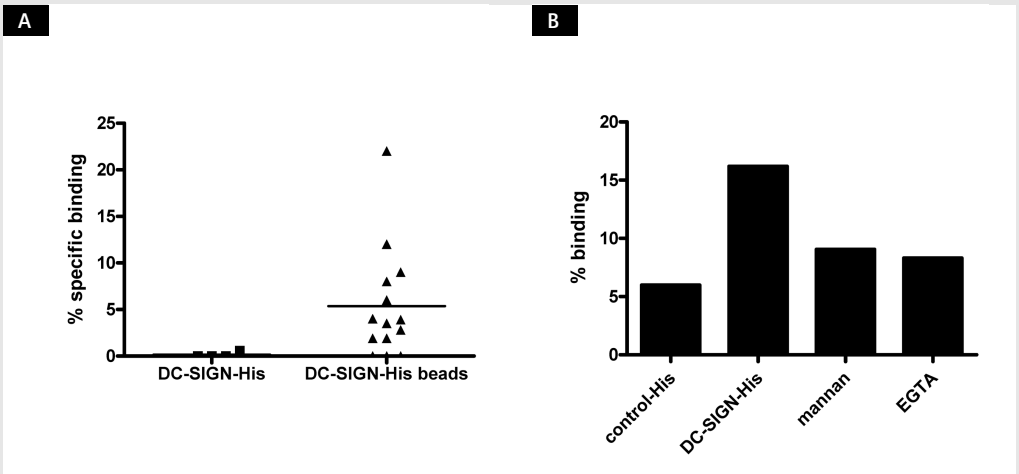


present in the PBL preparation, approximately 25% are able to bind DC-SIGN beads. All the different subsets contribute to the overall binding percentage of 20.7%. Similar results were obtained when positive and negative FACS-sorted T cells, B cells, and NK cells were analyzed for DC-SIGN binding (data not shown).

To determine whether ICAM-3 plays a role in the binding of soluble DC-SIGN-His-coated beads to PBL, binding studies were performed in the presence of ICAM-3-blocking mAbs, or by preblocking the DC-SIGN-His coated beads with soluble ICAM-3. Although blocking ICAM-3 hardly affected the percentage of PBL that bound DC-SIGN-His coated beads (data not shown), it reduced the MFI of the binding PBL by 35%. In addition, preblocking the

Figure 5

CHARACTERIZATION OF DC-SIGN-HIS BINDING TO PBL. (A) The binding of PBL to DC-SIGN-His construct, in free form or coated to streptavidin-coated fluorescent beads, was determined. PBL were allowed to bind 10 μg/ml recombinant soluble DC-SIGN-His construct for 30 min at 37°C, followed by incubation with Penta His Alexa-488 for 30 min at 37°C. For the beads assay, PBL were incubated for 30 min at 37°C with DC-SIGN-His-coated beads and subsequently analyzed by flow cytometry. Aspecific binding was determined by pretreatment of the DC-SIGN-His construct with 100 μg/ml mannan. Data are percentages of PBL isolated from several donors that displayed specific binding to the DC-SIGN-His construct. (B) Determination of aspecific binding of DC-SIGN-His beads to PBL by incubation cells with control-His beads or preincubation of DC-SIGN-His beads with 100 μg/ml mannan or 5 mM EGTA. One typical result out of 10 is depicted. (C) Characterization of DC-SIGN-His binding PBL by double/triple labeling FACS analysis. PBL isolated from a donor whose PBL showed high DC-SIGN-binding capacity were incubated with DC-SIGN-His-coated beads for 30 min at 37°C. Subsequently, the cells were labeled with FITC- and/or PE- conjugated mAbs for 20 min at RT where indicated. Samples were analyzed by flow cytometry, and FACS pictures are depicted. Left column represents FACS pictures with the specific cell populations gated (gate A, B or C with percentage positive cells) for testing binding capacity to DC-SIGN-His beads. Right columns represent binding results of the specific cell populations to DC-SIGN-His beads (percentage binding indicated in picture). SSC, Side-scatter; FSC, forward-scatter. (D) DC-SIGN-His beads were preincubated in binding buffer, or in binding buffer supplemented with 100 μg/ml soluble ICAM-3. The DC-SIGN-His beads preincubated in binding buffer were added to PBL, which were preincubated in binding buffer or in binding buffer supplemented with 20 μg/ml of the ICAM-3 blocking mAbs CBR3/1 and 3/2 (anti-ICAM-3 Ab). DC-SIGN-His beads preincubated in binding buffer supplemented with soluble ICAM-3 were added to PBL preincubated in binding buffer (soluble ICAM-3). Aspecific binding was determined by incubating PBL with DC-SIGN-His beads in the presence of 5 mM EGTA. PBL were allowed to bind DC-SIGN-His-coated beads for 30 min at 37°C. The mean cell fluorescence of DC-SIGN-His bead-binding PBL was determined by FACS analysis, and the percentages of the binding buffer control value were calculated. Data are mean ± SD of three experiments. Significant difference from binding buffer control, as determined by ANOVA and Dunnett's test: * $p < 0.05$. (E) The experiments shown in D were repeated using the T cell line HSB-2 instead of PBL. The mean cell fluorescence was determined by FACS analysis, and the percentages of the binding buffer control value were calculated. Data are mean ± SD of three experiments. Significant difference from binding buffer control, as determined by ANOVA and Dunnett's test: ** $p < 0.01$.



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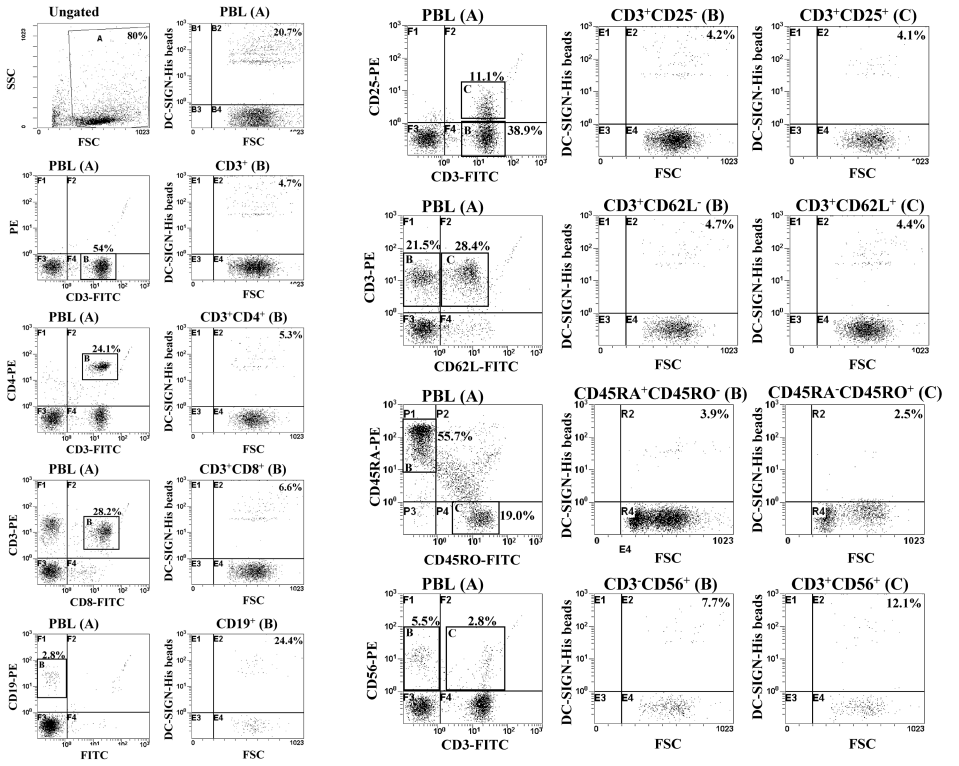

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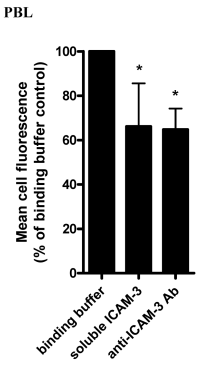

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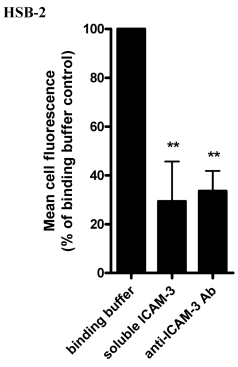


Table1

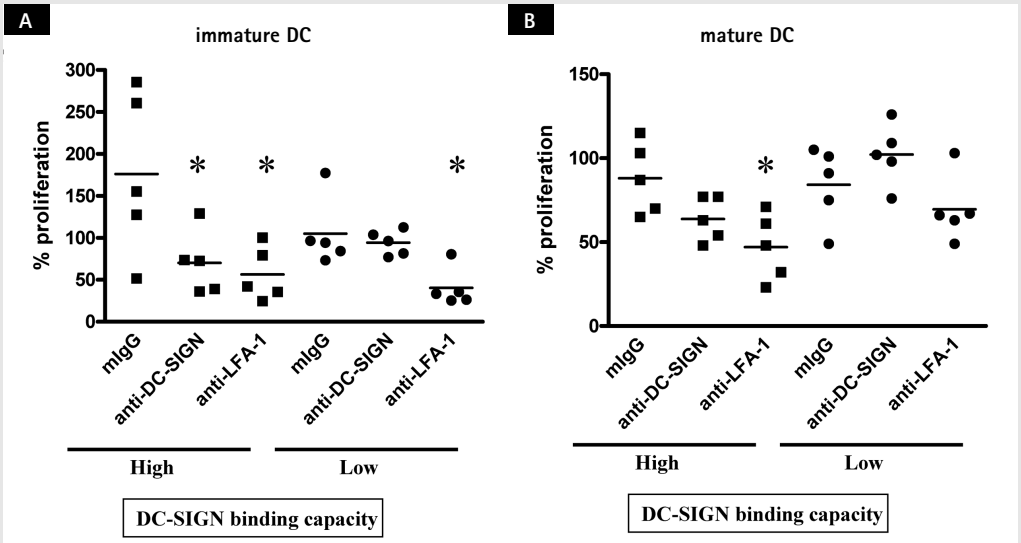
Cell population	% Binding
PBL total	20.7
T cells ($CD3^+$)	4.7
$CD3^+CD4^+$	5.3
$CD3^+CD8^+$	6.6
$CD3^+CD25^+$	4.1
$CD3^+CD25^-$	4.2
$CD3^+CD62L^-$	4.7
$CD3^+CD62L^+$	4.4
$CD45RA^+CD45RO^-$	3.9
$CD45RA^-CD45RO^+$	2.5
NK cells ($CD3^-CD56^+$)	7.7
NKT cells ($CD3^+CD56^+$)	12.1
B cells ($CD19^+$)	24.4

Table 1

CHARACTERIZATION OF DC-SIGN-HIS BINDING PBL BY DOUBLE/TRIPLE LABELING FACS ANALYSIS. PBL from the same donor as described in **Figure 5C** were incubated with DC-SIGN-His-coated beads for 30 min at 37°C. Subsequently, the cells were labeled with FITC- and/or PE- conjugated mAbs for 20 min at RT where indicated. Samples were analyzed by flow cytometry. Data are percentages of cells within the indicated cell populations that displayed binding to the DC-SIGN-His-coated beads.

Figure 6

BLOCKING EFFECTS WITH DC-SIGN MABS IN MLR AND WITH PBL SHOWING A HIGH DC-SIGN-HIS BINDING CAPACITY. CFSE-labeled PBL ($1x10^5$) from a donor whose PBL showed high DC-SIGN-His-binding capacity (22%, squares) or from a donor whose PBL showed low DC-SIGN-His-binding capacity (8%, circles) were cocultured with the same $1.5x10^3$ immature (A) or mature (B) allogeneic DC obtained from five different donors. After 7 days, proliferation was determined by flow cytometry. Control mouse IgG or blocking mAbs (10 µg/ml) directed against DC-SIGN (AZN-D1 and AZN-D3) or LFA-1 (NKI-L15) were added at the onset of the experiment. Data are percentages of control (no addition of mAbs). Each dot represents the average of one experiment performed in triplicate. Significant difference from control mouse IgG according to ANOVA, followed by the Student Newman Keuls test: * $p<0.05$.



DC-SIGN-His-coated beads with soluble ICAM-3 resulted in a 34% reduction in the MFI of the binding PBL **(Fig. 5D)**. As only a relatively low percentage of PBL bound DC-SIGN-coated beads, and the percentage of binding T cells in the PBL population was even lower **(Table 1)**, the T cell line HSB-2 was used as a model to determine whether ICAM-3 on the T cell interacts with the DC-SIGN-coated beads. On average, approximately 80% of HSB-2 cells bound DC-SIGN-coated beads (data not shown). Preblocking DC-SIGN-His-coated beads with soluble ICAM-3, as well as blocking ICAM-3 on the cell surface with mAbs, significantly reduced binding of the DC-SIGN-His beads to HSB-2 cells **(Fig. 5E)**.

Together, these data show that approximately 5% of PBL bind DC-SIGN beads and that binding is at least partly mediated by ICAM-3. The cells that bind DC-SIGN beads comprise all major cell subsets of PBL.

DC-SIGN-binding capacity parallels blocking capacity

As the number of PBL that bind DC-SIGN beads differs between donors, we tested whether this difference in binding could explain why some MLR are

blocked by mAbs against DC-SIGN, and others are not. PBL obtained from the donor characterized in **Table 1/Fig. 5C** and PBL from another donor showing lower DC-SIGN binding were cocultured with the same DC isolated from five different allogeneic donors. Based on the definitions given above and in the legend of **Figure 3B**, all the MLR belong to the low-responder category.

Upon stimulation with immature DC, PBL with high DC-SIGN binding capacity showed an overall decrease in T cell proliferation in the presence of blocking DC-SIGN and LFA-1 mAbs **(Fig. 6A)**. It has to be noted that addition of control mIgG itself influenced T cell proliferation. In contrast to the PBL showing a high DC-SIGN-binding capacity, DC-SIGN mAbs did not block proliferation of PBL with low DC-SIGN-binding capacity. Nevertheless, LFA-1 mAb blocked T cell proliferation considerably.

In MLR driven by mature DC, the presence of DC-SIGN mAbs also resulted in a decrease in overall T cell proliferation when PBL with high DC-SIGN-binding capacity were used **(Fig. 6B).** It is striking that PBL with low DC-SIGN-binding capacity displayed slightly enhanced T cell proliferation in the presence of DC-SIGN-blocking mAbs. The blocking effect of LFA-1 mAb on T cell proliferation is more pronounced in PBL with high DC-SIGN-binding capacity as compared to PBL with low DC-SIGN-binding capacity.

In conclusion, PBL isolated from the donor with high DC-SIGN-binding capacity were more prone to blocking by DC-SIGN mAbs in MLR than PBL from the donor with lower DC-SIGN-binding capacity.

Discussion

In the present study, we analyzed the relevance of DC-SIGN in DC-induced T cell proliferation. Our data indicate that its relevance in allogeneic T cell proliferation depends on the strength of the T cell response and on the percentage of PBL that are able to bind recombinant DC-SIGN.

A dependence on the strength of T cell response suggests an initiating role for DC-SIGN in DC-T cell interaction followed by other adhesive and costimulation events, that will boost T cell proliferation. The latter interaction is DC-SIGN-independent and therefore, not inhibitable by DC-SIGN mAbs. The height of a MLR response is directly proportional to the number of T cells that are activated by APC. The number of T cells that are activated is, besides efficient costimulation, dependent on the integrated outputs of all TCR signaling events per cell. Consequently, a weak MLR is observed when fewer cells are able to evoke sufficient TCR signaling events compared with a strong MLR. Sufficient TCR signaling is attained by clustering the TCR-MHC complexes, as the affinity of the TCR is very low [26]. Sustained signaling was also observed when the formation of the immunological synapse is inhibited [27]. DC-SIGN-ICAM-3 interactions prolong initial cell-cell contact and thereby prolong TCR signaling. Such prolonged cell-cell contact will result in a higher integrated signaling and thereby lowering the threshold for an immune response. Consequently, when more TCR-MHC are involved in the interaction, sufficient microclusters will be formed to initiate effective signaling and thereby minimizing the role for DC-SIGN-ICAM-3 interactions.

The proliferation data show that the degree of inhibition of T cell proliferation by LFA-1 and DC-SIGN blocking mAbs depends on the DC maturation state. Moreover, when using immature DC, DC-SIGN blocking mAbs only have an effect on some of the MLR that are categorized as weak MLR (<60% of PBL proliferate). Therefore, the variable blocking potential of DC-SIGN mAbs on the MLR, as shown in this study and by Granelli-Piperno *et al.* [16] is explained either by testing strong MLR or using mAbs that do not bind to the carbohydrate-recognition domain of DC-SIGN.

It is interesting that the degree of inhibition of T cell proliferation by LFA-1 mAb is also dependent on the strength of the T cell response. In MLR driven by mature DC, LFA-1 mAb could only block MLR with a weak response.

The kinetic-blocking experiments indicate that DC-SIGN and LFA-1 exert initial effects in DC-T cell communication, which are dominated readily by other adhesive and costimulatory mechanisms. Such a dominating effect will be more pronounced with mature DC in comparison with immature DC because of their stronger T cell stimulation capacity [28,29]. Indeed, relatively more low-responder MLR are observed when immature DC are used as T cell stimulators compared with mature DC as T cell stimulators **(Fig. 3B)**. In contrast to DC-SIGN and LFA-1 mAbs, T cell proliferation could be blocked over a long period of time by blocking CD6 mAb, which indicates CD6 plays an important and long-lasting role in DC-T cell contacts.

An initial effect of LFA-1 in DC-T cell communication is supported by a study of Bachmann *et al.* [30]. They demonstrate that LFA-1 induces T cell activation by promoting adhesion of T cells to APC instead of providing long-lived costimulatory signals [30]. Another study also indicates that LFA-1 is not able to provide strong costimulatory signals such as CD28, which is the most dominant costimulatory molecule [31].

The dominating role of other adhesive and costimulatory mechanisms may

play a minor role when less professional APC are used such as THP-1 cells differentiated into DC-SIGN$^+$ cells. These DC-like cells induce T cell proliferation, which is blocked by DC-SIGN mAbs [13]. Probably, the effect of DC-SIGN on T cell proliferation is more easily over-ruled than that of LFA-1 because of the transient nature of the DC-SIGN/ICAM-3 interaction. It has been reported that DC-SIGN/ICAM-3-dependent adhesion between DC and T cells varies and reaches an optimum after 20 min [7]. In addition, DC-SIGN can recruit LFA-1 to the contact site and shift from initial transient DC-SIGN-ICAM-3 interactions to more stable LFA-1-ICAM-3 interactions [25]. Furthermore, ICAM-3 is a dynamic molecule, as it is recruited rapidly to the APC-T cell contact site followed by a redistribution to the outer zone of the cell-cell interface upon contact stabilization [4].

In agreement with previous studies [32,33], our DC-T cell conjugates do not show an enriched distribution of DC-SIGN in the DC-T cell contact area, regardless of the use of immature or mature DC and of the distribution pattern of ICAM-3. Apparently, sufficient DC-SIGN molecules are present on the DC membrane to establish a transient interaction with ICAM-3.

Funatsu *et al.* [34] reported that ICAM-3 molecules isolated from human PBL only contain 6% high mannose-type oligosaccharides of the total ICAM-3 oligosaccharide pool. The authors of this study suggest that these high mannose-type oligosaccharides are expressed on a special subset of T cells [34]. However, we could not pinpoint the DC-SIGN-binding cells to one specific T cell population. Instead, we observed that all cell populations analyzed bind DC-SIGN to a similar degree, except for B cells, which showed a much higher specific binding (**Table 1**). Thus, besides major T cell subsets, DC-SIGN beads also bind B cells and NK cells. Interactions between DC and B cells and DC and NK cells modulate DC function and can indirectly have an impact on T cell response [35,36].

It seems likely that high mannose-type oligosaccharides are expressed at low levels on all T cells and underlie the transient binding of DC-SIGN to T cells. This concept is supported by the finding that DC-SIGN-His-coated beads were superior in binding to PBL as compared with soluble DC-SIGN-His. Multiple DC-SIGN-His molecules are attached to each fluorescent bead, endowing it with a high avidity, thus improving stable interactions between the bead and the low number of specifically glycosylated ICAM-3 molecules expressed on PBL. DC-SIGN–His, in soluble form, may at best form tetramers [37] and thus, will have a lower avidity.

PBL isolated from some donors showed a considerable higher binding to DC-SIGN-His-coated beads than other donors. This high-binding potential was consistent over time, as PBL with a high DC-SIGN-binding capacity from a specific donor still showed a high specific binding to DC-SIGN-His-coated beads when measured 6 and 18 months later (data not shown).

Snyder *et al.* [38] found submicromolar binding affinities when using ICAM-3, which was produced in the mouse cell line NSO. Glycosylation is important for reactivity of DC-SIGN, as Snyder *et al.* [38] show in their paper for gp120, the other ligand of DC-SIGN. Mouse cells are known to glycosylate incompletely *in vitro*-produced proteins. As DC-SIGN recognizes high mannose-type oligosaccharides, it is not surprising that those authors find submicromolar-binding affinities. The ligand is simply not expressed at sufficient levels. As reported above, ICAM-3 isolated from human PBL is expressing only 6% high mannose-type oligosaccharides [34]. Moreover, the same authors show that for binding, tetrameric DC-SIGN is adamant [38]. Chimeric Fc-ICAM-3 is believed to be dimeric, which adds to their poor binding results.

Geijtenbeek *et al.* [7] used beads that harbor multiple Fc-ICAM-3 ligands for binding studies to circumvent those valency issues. It seems likely that the donors whose PBL bind DC-SIGN with high capacity express an increased level of specifically glycosylated ICAM-3 and possibly other specifically glycosylated ligands. Changes in glycosylation can be related to the differentiation status of a cell, as a T cell displays several glycosylation patterns during its development [39]. Ryan *et al.* [19] showed a preferential binding of naïve $CD45RA^+$ cells to DC-SIGN-His. However, in our study no clear differences were observed between naïve/resting cells ($CD45RA^+$, $CD62L^+$) and memory/activated cells ($CD45RO^+$, $CD25^+$) [40-42].

PBL, with a high DC-SIGN-binding capacity, were more prone to DC-SIGN-blocking mAbs in the MLR, even when mature DC were used to stimulate the T cells. In these MLR, DC-SIGN can bind firmly to a higher percentage of T cells and may have a prolonged effect on these cells compared with cells that bind transiently. In a MLR, only 1 to 10% of the T cells are able to respond to the allogeneic MHC-peptide complexes expressed on DC. Therefore, only a relatively small number of T cells binding firmly to DC-SIGN might be required to have a substantial effect in the MLR. Because of the higher frequency of firm binding cells in the donor whose PBL have a high DC-SIGN-binding capacity, the effect of blocking DC-SIGN in the MLR is observed more rapidly. Accordingly, the level of DC-SIGN-binding capacity of PBL may explain why not all weak MLR stimulated by immature DC are down-regulated by DC-SIGN-blocking mAbs.

The MLR experiments in **Figure 6** show that the inhibitory action of DC-SIGN mAbs is not DC donor-specific or dependent on level of DC-SIGN expression. This can be concluded from the fact that the use of the same DC donor and therefore, the same DC-SIGN expression level, in combination with two PBL donors, resulted in different effects of the DC-SIGN mAbs.

Our data indicate that DC-SIGN is involved in early contacts between DC and T cells. It is tempting to speculate that DC-SIGN is involved in the initial exploratory contact of T cells with DC in a similar way as described for ICAM-3 [4]. In this regard, DC-SIGN may act like a selectin by mediating the 'rolling' of T cells over DC in a similar way as selectins do by mediating the rolling of T cells along the endothelium [43]. Future dynamic studies performed in a three-dimensional setting could address this phenomenon.

Summarizing, this study indicates an initial and transient role for DC-SIGN in T cell proliferation, which becomes apparent in weak MLR and when the percentage of DC-SIGN binding PBL is high.

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Chapter 5

Binding of the adhesion and pathogen receptor DC-SIGN by monocytes is regulated by the density of Lewis X molecules

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Abstract

Soluble DC-SIGN (CD209) bind unsialylated Lewis X epitopes that are abundantly expressed on neutrophils. Due to the low expression of unsialylated Lewis X epitopes on monocytes, no binding of soluble DC-SIGN molecules was seen. In contrast, beads coated with multiple DC-SIGN molecules show a high percentage of binding to monocytes. The increased number of DC-SIGN molecules present on the beads enable multivalent interactions between the DC-SIGN molecules and the scarce Lewis X epitopes present on monocytes. Increased expression of unsialylated Lewis X epitopes on monocytes after neuraminidase treatment coincided with enhanced binding to soluble DC-SIGN. Multiple unsialylated Lewis X epitopes in close proximity of each other are now able to interact multivalently to soluble DC-SIGN. From these findings, we conclude that firm interactions between DC-SIGN and monocytes can be established by either increasing the density of DC-SIGN molecules at the cell surface or by increasing the number of Lewis X epitopes. Regulating the number of ligands endows monocytes with the capacity to modulate binding to DC-SIGN. This may result in a bi-directional cross-talk between DC and monocytes, to modulate innate and/or adaptive immune responses.

Introduction

Dendritic cell specific ICAM-3 grabbing non-integrin (DC-SIGN) belongs to the C-type lectin family and is expressed on dendritic cells (DC) and certain macrophages [1-3]. It acts as adhesion and pathogen recognition receptor by recognizing a broad array of ligands which have high-mannose residues or fucose residues in common [4,5]. The DC-SIGN molecule forms tetramers endowing the molecule with multiple binding sites to its ligands [6]. On DC, DC-SIGN is organized in microdomains of 200 nm in diameter that enable binding to small virus-sized particles [7]. Based on these findings, oligomeric DC-SIGN molecules in close proximity of each other are adamant to establish firm multivalent interactions with its ligands. Those ligands have to be properly spaced to enable multivalent binding [8]. Soluble DC-SIGN is able to bind to neutrophils in a MAC-1 dependent manner [9]. Here, the binding of soluble DC-SIGN as well as multivalent DC-SIGN to monocytes is reported. Moreover, sialic acid residues were removed from monocytes by treatment with neuramidase to reveal putative DC-SIGN binding entities.

Materials and Methods

Production of DC-SIGN beads

DC-SIGN-His [10] or control-His (six-His tagged humanized single chain h5G1.1 antibody [11]) were coated onto carboxylate-modified TransFluo-Spheres (488/645 nm, 1.0 µm; Molecular Probes, Leiden, The Netherlands [12]) First, beads coated with streptavidin were incubated with biotinylated horse-anti-mouse IgG (Vector, Brunschwig Chemie, Amsterdam, the Netherlands) at 37°C for 2 h followed by an overnight incubation with mouse-anti-Penta-His (Qiagen, Benelux B.V., Venlo, The Netherlands) at 4°C. Subsequently, the beads were incubated with His-constructs at 4°C for two days.

Determination of the specificity

Specificity was determined by preincubation with 100 µg/ml mannan in binding buffer (20 mM Tris-HCl, pH 8.0, 150 mM NaCl, 1 mM $CaCl_2$, 2 mM $MgCl_2$ and 0.5% BSA) at room temperature for 20 min. Subsequently, 5×10^4 PBMC were added to the beads and incubated at 4°C for 30 min. After washing, adhesion of the beads to the cells was assessed by flow cytometry.

Isolation of $CD14^+$ monocytes

$CD14^+$ cells were isolated from PBMC by performing positive selection with $CD14^+$ micro magnetic beads (Miltenyi Biotec., Bergisch-Gladbach, Germany) according to the manufacturer's instructions.

Removal of sialic acid residues

$CD14^+$ monocytes (2×10^6 cells/ml in PBS) were incubated with 20×10^{-3} units/ml neuraminidase (type V, from *Clostridium perfringens,* Sigma) at 37°C for 10 min.

Electron microscopic analysis

TEM labeling was performed as described previously [7]. Briefly, monocytes were labeled with isotype control (mIgM, Ancell, Kordia BV, Leiden, The Netherlands) or anti-CD15 (clone 80H5, Immunotech) followed by incubation with rabbit anti-mouse IgM (µ chain specific, Jackson Immunoresearch, Brunschwig Chemie B.V., Amsterdam, The Netherlands) and a final incubation with Protein A gold 10 nm diameter (kind gift of M. Wijers and H. Croes, Nijmegen Centre for Molecular Life Sciences). Specimens were observed in a transmission electron microscope (model 1010; JEOL), operating at 60–80 kV.

Binding of soluble DC-SIGN

Neuraminidase-treated and untreated monocytes (5×10^4) were incubated with soluble DC-SIGN-Fc (10 µg/ml, [13]) in binding buffer at 4°C for 30 min. Where indicated, DC-SIGN was preincubated with 100 µg/ml mannan or 5 mM EGTA prior to the binding assay at room temperature for 20 min. Subsequently, the samples were incubated with FITC-conjugated goat-anti-human Fc (Cappel, Irvine, CA, USA) at 4°C for 30 min.

Results

The spatial requirements for binding were unravelled using oligomeric soluble DC-SIGN molecules and multivalent beads coated with DC-SIGN. Binding of DC-SIGN beads to PBMC was evident from an increased side scatter of the monocyte population **(Fig. 1A)**. Control beads lack and blocking with mannan abolished this increase in side scatter. After subtracting the binding in the presence of mannan, the latter representing non-DC-SIGN mediated binding, 45% of the monocytes bound to DC-SIGN beads **(Fig. 1B)**. Isolation of monocytes with the aid of $CD14^+$ MicroBeads showed a similar binding percentage whereas only 3% of the $CD14^-$ cells bound the DC-SIGN beads **(Fig. 1C)**. Thus, demonstrating monocyte specific binding.

Recently, it was reported that soluble DC-SIGN binds neutrophils via Mac-1 that expresses Lewis X epitopes [9]. In contrast, monocytes did not bind soluble DC-SIGN due to lack of Lewis X expression [9]. However, we now observed that monocytes did show low Lewis X expression when using an-

Figure 1

DC-SIGN BEADS BIND TO MONOCYTES. (A) Scatterplots and (B) histogram profiles of PBMC binding to coated beads. (A) gate A represents free beads, gate B: lymphocytes, and gate C: monocytes. Percentage of gated monocytes (gate C) that have bound beads is depicted in (B). (C) Binding of DC-SIGN beads to $CD14^+$ and $CD14^-$ cells as determined with the flowcytometer. Where indicated, beads were preincubated with 100 µg/ml mannan, 5 mM EGTA or 20 µg/ml AZN-D1 [1]. One representative experiment out of 5 is depicted.

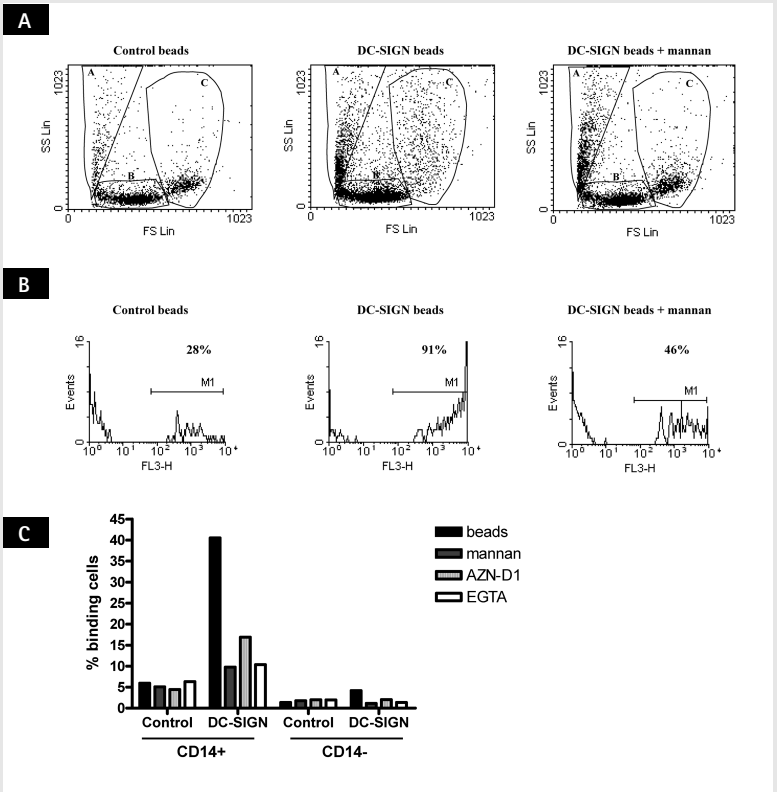
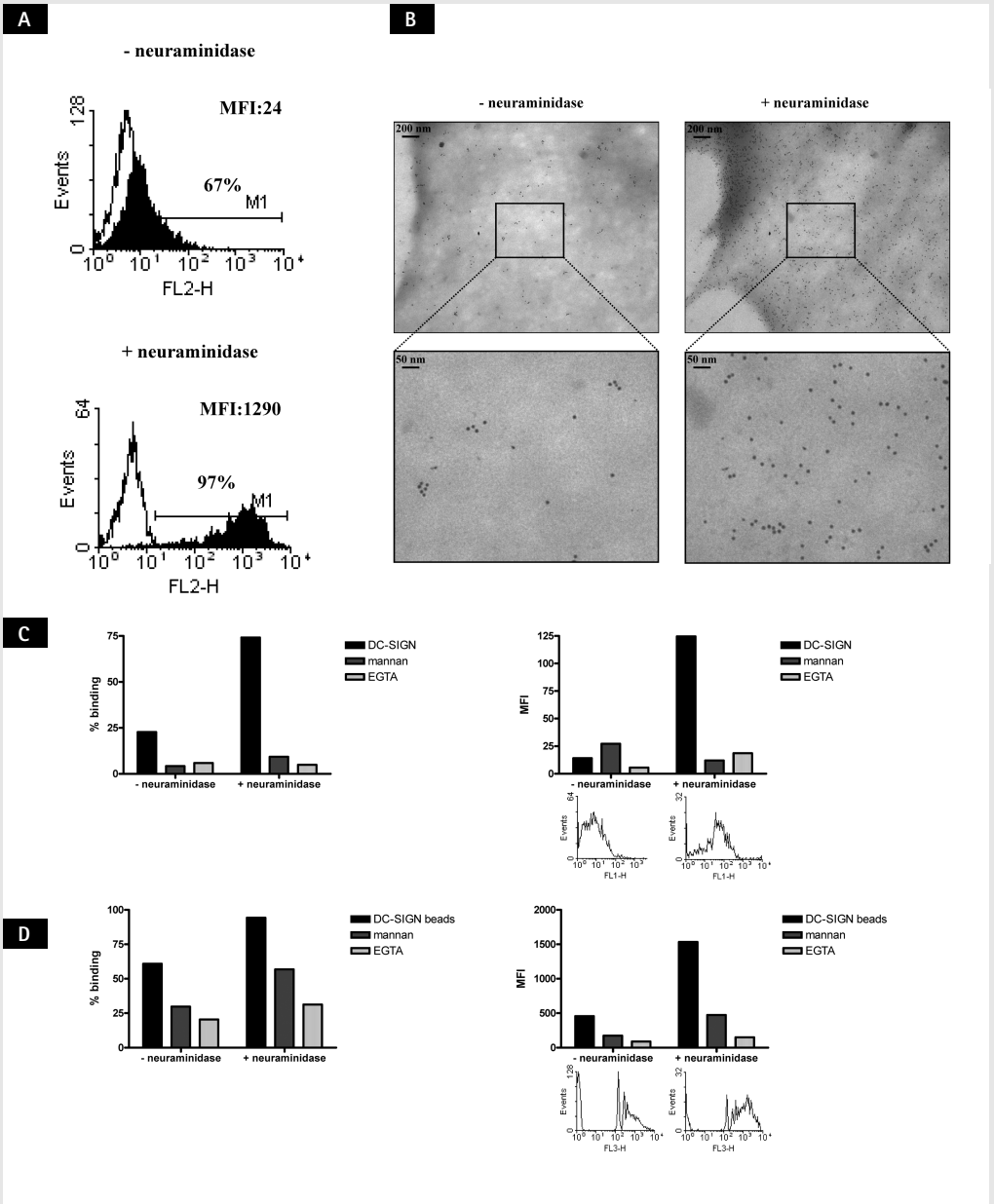


Figure 2

INCREASED EXPRESSION OF LEWIS X BY MONOCYTES RESULTS IN INCREASED BINDING TO DC-SIGN. (A) CD15-PE (clone 80H5; Immunotech, Beckman Coulter B.V., Mijdrecht, The Netherlands) staining of neuraminidase-treated and untreated monocytes. MFI of Lewis X (CD15) expression and percentage of positive cells of 1 representative experiment out of five are depicted. (B) TEM pictures of Lewis X expression on monocytes untreated or treated with neuraminidase. (C) Flowcytometric analysis of soluble DC-SIGN binding to untreated and neuramidase-treated monocytes. Percentage of cells that have bound DC-SIGN (left panel), MFI (right panel) and FACS histograms of one representative experiment out of five are depicted. (D) Flowcytometric analysis of DC-SIGN coated beads binding to untreated and neuramidase-treated monocytes. Percentage of cells that have bound DC-SIGN (left panel), MFI (right panel) and FACS histograms of one representative experiment out of four are depicted.

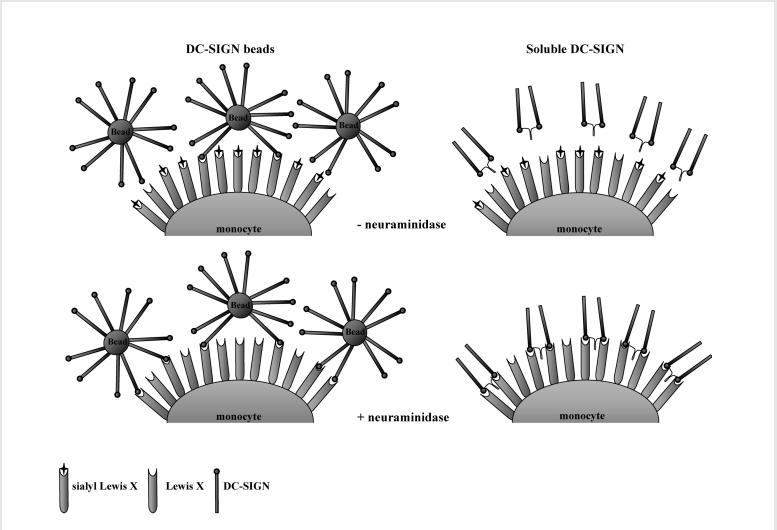


other anti-Lewis X antibody **(Fig. 2A)**. This is in agreement with a study of Nakayama *et al.* [14] who reports that the lower but significant expression level of Lewis X on monocytes compared to neutrophils is due to exclusive expression of the potent Lewis X-generating enzyme α1,3-fucosyltransferase IX in neutrophils.

This notion was further substantiated by the finding that monocytes after treatment with neuraminidase, to remove terminal sialic residues of various glycomolecules, showed a significant increase in Lewis X expression **(Fig. 2A)**. This indicates that Lewis X epitopes on monocytes are predominantly masked by sialyl groups as also reported by Ohmori *et al.* [15]. To visualize the distribution of Lewis X, transmission electron microscopy (TEM) pictures of monocytes were made before and after neuraminidase treatment **(Fig. 2B)**. Before neuraminidase treatment, only a few randomly spaced Lewis X epitopes were observed on the monocyte membrane. After neuraminidase treatment the distribution, albeit still random, showed a significant increase in the number of Lewis X epitopes as is also evident from the FACS staining profiles. Interestingly, treatment of monocytes with neuraminidase also resulted in an increased specific binding to soluble DC-SIGN **(Fig. 2C)**. This increased binding is also observed with DC-SIGN beads and is reported by the mean fluorescence intensity (MFI) **(Fig. 2D)**. The increased MFI demonstrates that per cell more DC-SIGN beads bind to neuraminidase-treated monocytes when compared to untreated monocytes. The discrepancy between DC-SIGN beads and soluble DC-SIGN in binding to monocytes can be explained by the fact that beads of 1 μm in diameter have a large interaction surface saturated with numerous DC-SIGN molecules that can engage simultaneous interactions with several spatially dispersed unsialylated

Figure 3

MULTIVALENT INTERACTIONS ENHANCE BINDING BETWEEN DC-SIGN AND MONOCYTES.
Beads saturated with numerous DC-SIGN molecules that engage simultaneous interactions with several individual unsialylated Lewis X molecules. In contrast, when soluble DC-SIGN is used, the contact surface and therefore, the number of interacting unsialylated Lewis X molecules is much smaller. For that reason, stable interactions only form between soluble DC-SIGN and monocytes when the number of unsialylated Lewis X are increased by neuraminidase treatment.



Lewis X epitopes **(Fig. 3)**. This results in a stable binding to monocytes whereas soluble DC-SIGN can not establish a stable binding to monocytes because of a smaller contact surface and, therefore, the number of interacting molecules remains low. For that reason, only stable interactions between soluble DC-SIGN and monocytes are formed when the number of unsialylated Lewis X molecules is increased by neuraminidase treatment.

Discussion

A difference in multivalent presentation of DC-SIGN molecules and its glycosylated ligand is sustained by the glycan array studies of Guo *et al*. [4] and Blixt *et al*. [16]. In these studies, it was reported that soluble DC-SIGN-Fc is less capable of binding a series of natural branched high-mannose N-glycans than tetrameric recombinant DC-SIGN. This may be explained by the valency of the DC-SIGN molecules (dimeric DC-SIGN-Fc vs tetrameric DC-SIGN) and the difference in density of the coated glycans [16]. Also Snyder *et al*. [6] reported that a multivalent presentation of DC-SIGN molecules is important for its binding potential. These authors showed that tetrameric DC-SIGN had a substantial increased avidity for the HIV-1 envelope glycoprotein gp120 compared to monomeric DC-SIGN [6].

Approximately 40% of the untreated monocytes could bind to the DC-SIGN beads. As human blood monocytes consist of a heterogeneous population this finding may indicate that DC-SIGN beads bind to a subpopulation of monocytes [17]. Obviously, this can be related to expression levels of Lewis X. Interestingly, expression of Lewis X seems to be associated with the activation status of the monocyte as Lewis X expression is increased by pro-inflammatory cytokines as well as LPS [18,19].

While the physiological significance of DC-SIGN interacting with monocytes remains to be determined, it is tempting to speculate that DC-SIGN expressed on DC mediates bidirectional cross-talk between DC and monocytes. This may for instance result in activation of monocytes upon engagement of Lewis X by DC-SIGN like is shown for several Lewis X antibodies [20]. Activated monocytes may, in a similar way as described for activated neutrophils [9], induce DC maturation via delivery of TNF-α to DC in DC-monocyte contacts. Moreover, interactions between DC and monocytes may result in antigen transfer as reported for macrophages and DC [21]. Monocytes transfer antigens, taken up in the periphery, to lymph node resident DC that will on their turn induce an effective primary immune response against these antigens. Interactions between DC and monocytes may occur in inflamed peripheral lymph nodes as large numbers of monocytes are recruited to these sites [22]. Moreover, interactions between DC and monocytes may occur in peripheral tissues once monocytes enter peripheral tissues to differentiate into macrophages or DC [23,24]. Possibly, communication with DC in peripheral tissues control the differentiation fate of the monocyte in addition to cytokines and environmental factors [25].

In summary, this study shows that firm interactions between DC-SIGN and monocytes can be established by increasing the number of DC-SIGN molecules or the number of Lewis X epitopes. As expression levels of DC-SIGN on DC and Lewis X on monocytes are subject to activation stimuli this may have important physiological implications on the innate and adaptive immune system [1,18,19,26].

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Chapter 6

Aberrant glycosylation of leukemic cells enhances binding to the immune response modifiers DC-SIGN and L-SIGN

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Abstract

Background and objectives. DC-SIGN and L-SIGN recognize carbohydrates expressed on pathogens and cells. These C-type lectins are expressed on dendritic cells (DC) and/or liver sinusoidal endothelial cells (LSEC) which can modulate immune responses. In acute lymphoblastic leukaemia (ALL), aberrant glycosylation of blast cells may alter their interaction with DC-SIGN and L-SIGN and thereby affecting their immunological elimination.
Design and Methods. Recombinant DC-SIGN and L-SIGN were coated to fluorescent beads and adhesion to blood and bone marrow cells from B- and T-ALL patients was assessed by flow cytometry. Peripheral blood lymphocytes from healthy donors were included as control.
Results. Overall, increased binding of ALL cells to DC-SIGN and L-SIGN was observed compared to cells from healthy donors. L-SIGN bound a higher percentage of leukemic and normal cells than DC-SIGN. The highest percentage of binding to L-SIGN was found for B-ALL bone marrow cells. DC-SIGN bound equally to B-ALL and T-ALL cells. Within ALL subtypes, DC-SIGN binding was higher with mature T-ALL. Interestingly, our data demonstrate that increased binding of DC-SIGN and L-SIGN to peripheral blood cells from B-ALL patients is associated with poor survival.
Interpretation and Conclusions. These data demonstrate that ALL cells have an aberrant glycosylation compared to their normal counterparts. Moreover, high binding of B-ALL peripheral blood cells to DC-SIGN and L-SIGN correlates with poor prognosis. Apparently, when B-ALL cells enter the blood circulation and are able to interact with DC-SIGN and L-SIGN the immune response is shifted towards tolerance. These findings open prospects for DC-SIGN and L-SIGN as novel prognostic and therapeutic tools in ALL.

Introduction

Acute lymphoblastic leukaemia (ALL) is an uncontrolled proliferation of lymphoblasts committed to either the B-cell (B-ALL) or the T-cell (T-ALL) lineage. The cells fail to differentiate and accumulate in the haematopoietic tissues [1]. In childhood ALL is the most frequent acute leukaemia and children have a much better prognosis than adults diagnosed with ALL. This is explained by the higher frequency of high-risk ALL with greater drug resistance in adults but also due to poorer tolerance of intensive treatment schemes in adults [2]. Identification of prognostic factors to stratify into standard-, intermediate-, and high-risk forms and adaptation of the chemotherapy regimens has improved the prognosis of ALL in children during the last three decades [3]. Risk classification in ALL is based on several factors like age, leukocyte count, cytogenetics, and ALL lineage [4,5]. Since a role for aberrant glycosylation in cancer is more and more appreciated [6], we hypothesize that the glycosylation status of ALL might be a prognostic factor.

Glycosylation is frequently altered in tumor cells compared to their normal counterparts. This altered glycosylation is usually the result of differences in expression levels of glycosyltransferases that catalyze the stepwise synthesis of both N- and O-linked oligosaccharides [7,8]. An aberrant glycosylation pattern can be beneficial for the tumor cell because it enables migration and metastasis as well as escape from immune surveillance [8]. Glycosylation patterns are recognized by C-type lectins, that bind carbohydrate structures in a Ca^{2+}-dependent manner [9]. In the immune system, C-type lectins act as adhesion and/or as pathogen recognition receptors [10]. As pathogen recognition receptor, C-type lectins recognize specific carbohydrate moieties that are present on pathogens and play an important role together with other receptors in the immune defense against pathogens [11,12]. As adhesion receptors, C-type lectins mediate interactions between different haematopoietic cells [11]. Since the C-type lectin DC-SIGN mediates dendritic cell (DC)-T cell interactions by binding to T cells [13], DC-SIGN is a prime candidate to test differential binding to peripheral blood lymphocytes (PBL) from healthy donors and their malignant counterparts from ALL patients. DC-SIGN is expressed on dendritic cells and certain macrophages and besides functioning as adhesion receptor it also acts as pathogen recognition receptor by recognizing a variety of microorganisms [10]. The ligands for DC-SIGN contain high-mannose or fucose residues [14]. L-SIGN (CD299) is a close homologue of DC-SIGN that shares 77% homology in amino acid sequence [15,16]. This C-type lectin is expressed on sinusoidal endothelial cells in liver and lymph nodes [16]. Liver sinusoidal endothelial cells (LSEC) have been implicated in tolerance induction [17,18]. L-SIGN also recognizes high-mannose residues, but it does not have a similar fucose-binding specificity [14,19]. The tolerance inducing capacity of the cells that express L-SIGN prompted us to study the binding to L-SIGN besides binding to DC-SIGN. Since binding to DC-SIGN is dependent on the valency of the interacting partners [20], beads coated with multiple DC-SIGN and L-SIGN molecules were used throughout the study.

This study demonstrates increased binding of recombinant DC-SIGN and L-SIGN to cells isolated from T- and B-ALL patients when compared to PBL from healthy donors. This indicates an altered glycosylation pattern on ALL cells exemplified by the increased expression of Lewis X (CD15). In B-ALL patients, increased binding of DC-SIGN and L-SIGN to peripheral blood cells is correlated with a shorter relapse-free-survival.

Materials and Methods

Cells

PBL from healthy donors were isolated from buffy coats of volunteers by Ficoll density centrifugation. After a 1 h adherence step to plastic the non-adherent cells (PBL) were collected. ALL samples were obtained from untreated T- and B-lineage ALL patients that were 2 to 64 years of age at the time of diagnosis. Diagnosis was based on morphological/cytochemical evaluation according to standard French-American-British criteria as well as by immunophenotyping using a panel of monoclonal antibodies. Mononuclear cell fractions were isolated from peripheral blood (PB) or bone marrow (BM) samples by Ficoll density gradient centrifugation. The ALL T cell line HSB2 was obtained from ATCC (CCL-120.1) and cultured in Iscove's modified Dulbecco's medium containing 5% FCS.

Recombinant His constructs

Recombinant DC-SIGN- and L-SIGN-His consist of the extracellular domain of DC-SIGN or L-SIGN harboring a six-His-tag at the N-terminus. The DNA construct of DC-SIGN-His was kindly provided by Dr. Clark [21]. L-SIGN-Fc construct was kindly provided by Dr. Kretz-Rommel [22]. The following primers were used to make the L-SIGN-His construct using the pQE30-vector (Qiagen, Chatsworth, CA); *Bam*H1, 5'-acgacgggatcctccaaggtccccagctcc-3'; and *Hin*DIII, 5'-acgacgaagcttctattcgtctctgaagcaggctg-3'. Bacterial strain *E. coli* M15(Prep4) was transfected with the DC-SIGN- or L-SIGN-His constructs and expression was induced by 0.1 mM IPTG (Sigma, Sigma-Aldrich Chemie B.V. Zwijndrecht, the Netherlands). The inclusion bodies were collected and the protein was solubilized in 8.5 M Urea and subsequently refolded by stepwise dialysis against buffers containing decreasing Urea concentrations. The protein preparation was incubated with mannan agarose beads (Sigma). Only properly refolded and therefore functional protein is able to bind to mannan agarose beads. After several washing steps to remove unbound protein the functional DC-SIGN and L-SIGN were eluted from the beads by EGTA that removes the Ca^{2+}-ion from DC-SIGN and L-SIGN that is essential for proper binding. The six-His tagged humanized single chain h5G1.1 antibody was used as control and kindly provided by Dr. Kretz-Rommel [23].

Coating of fluorescent microspheres with recombinant His constructs

Control-His, DC-SIGN–His, and L-SIGN-His were coated onto streptavidin coated carboxylate-modified TransFluoSpheres (488/645 nm, 1.0 µm; Molecular Probes, Leiden, The Netherlands [24]). First, streptavidin-coated beads were incubated with biotinylated horse-anti-mouse IgG (10 µg; Vector, Brunschwig Chemie, Amsterdam, the Netherlands) at 37°C for 2 h followed by an overnight incubation with mouse-anti-Penta-His (1 µg; Qiagen, Benelux B.V., Venlo, The Netherlands) at 4°C. Subsequently, the beads were loaded with 250 ng recombinant His constructs at 4°C for two days.

Fluorescent beads adhesion assay

The DC-SIGN, L-SIGN and control beads were incubated with 10^5 cells in binding buffer (20 mM Tris-HCl, pH 8.0, 150 mM NaCl, 1 mM $CaCl_2$, 2 mM $MgCl_2$ and

0.5% BSA) in a 96-wells V-shaped bottom plate at 37°C for 30 min. Where indicated, the beads were pre-incubated with 100 µg/ml mannan or 5 mM EGTA prior to the binding assay at room temperature (RT) for 20 min. After washing, binding of the beads to the cells was assessed by flow cytometry.

Characterization of DC-SIGN- and L-SIGN-binding cells by flow cytometry

Cells were incubated with DC-SIGN, L-SIGN, or control beads as described above. After washing, the cells were labeled for 20 min at RT with combinations of either CD5-PE (clone BL1a; Immunotech, Beckman Coulter B.V., Mijdrecht, The Netherlands) or CD7-FITC (clone 8H8.1; Beckman Coulter, Beckman Coulter B.V., Mijdrecht, The Netherlands) or CD10-FITC (clone ALB1; Beckman Coulter), and CD34-PE (clone 581; Beckman Coulter). Samples were analyzed by flow cytometry (Cytomics Fc 500, Beckman Coulter, Fullerton, CA).

FACS staining

ALL cells were labeled for 20 min at RT with either CD15-PE (clone 80H5; Immunotech), or CD24-FITC (clone CLB-gran-B-ly/1, 1B5; PeliCluster, Sanquin, Analis SA/N.V., Namen, Belgium), or CD7-FITC (clone 8H8.1; Beckman Coulter). Samples were analyzed by flow cytometry.

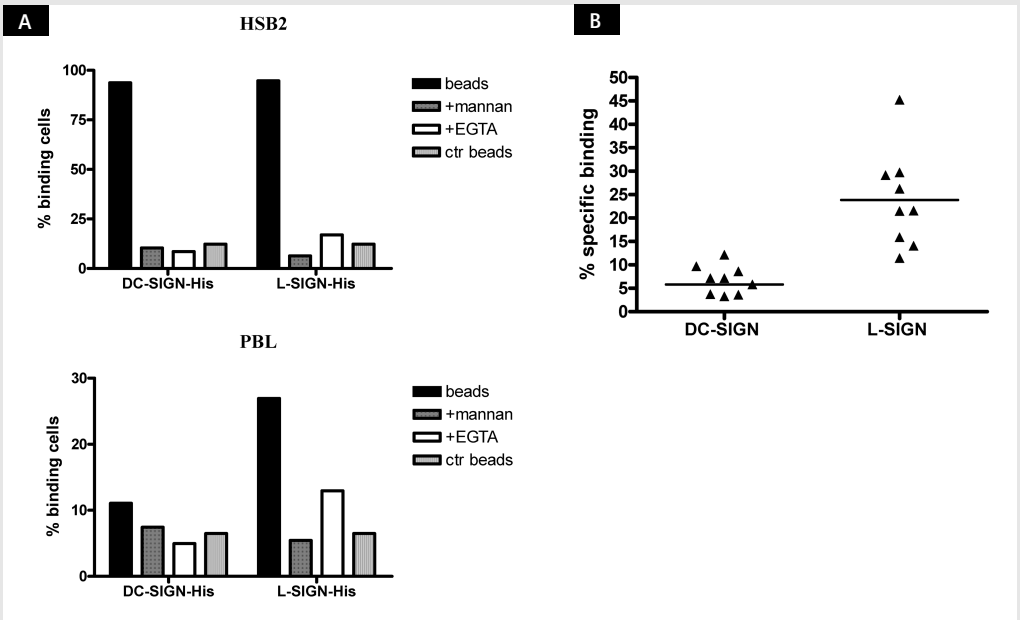
Results

Differences in binding capacity of DC-SIGN and L-SIGN to HSB2 and PBL

The large difference in binding capacity of HSB2 and PBL to DC-SIGN and L-SIGN beads was the incentive to study the binding capacity of other ALL samples. The HSB2 cell line was originally derived from a T-ALL patient, whereas PBL comprise mainly T cells (±73%), B cells (±7%) and NK cells (±5%) [25]. Both C-type lectins bind over 90% of the HSB2 cells whereas a much lower specific binding to PBL was observed **(Fig. 1A)**. Binding to HSB2 and PBL is blocked by both mannan and EGTA, indicating that the binding is C-type lectin dependent. Remarkably, the binding capacity of L-SIGN beads to PBL is higher than found for DC-SIGN beads. DC-SIGN beads bind to 1-15% of PBL from normal donors whereas L-SIGN beads bind 10 to 45% of PBL **(Fig. 1B)**. The substantial higher binding of HSB2 cells to both lectins indicates aberrant glycosylation of the HSB2 cells when compared to normal T cells.

Figure 1

DIFFERENCES IN BINDING CAPACITY OF DC-SIGN AND L-SIGN TO HSB2 AND PBL. (A) The binding of HSB2 cells and PBL by DC-SIGN- and L-SIGN-His constructs coated to fluorescent beads was determined. A control-His construct was included to determine aspecific binding. The cells were incubated for 30 min at 37°C with the His-construct coated beads and subsequently analyzed by flow cytometry. Aspecific binding was also determined by pretreatment of the DC-SIGN- and L-SIGN-His constructs with 100 µg/ml mannan or 5 mM EGTA. Data are percentages of cells that displayed binding to the DC-SIGN- and L-SIGN-His constructs. (B) Overview of specific binding (determined by subtracting background binding obtained in the presence of mannan) of PBL isolated from several donors to DC-SIGN- and L-SIGN-His coated fluorescent beads.



Increased binding of DC-SIGN to ALL cells

The binding of DC-SIGN beads to patient T-ALL cells was assessed to unravel a putative common denominator for ALL cells. If the same glycosylation pattern as found for the HSB2 cells is found in all T-ALL cells, the same high binding is expected. ALL cells committed to the B cell lineage (B-ALL) were taken along. Compared to PBL from healthy donors, an increased binding was observed for DC-SIGN beads to T-ALL and B-ALL cells **(Fig. 2A)**. However, the binding was much lower than was observed for the T-ALL cell line HSB2. No significant differences in binding to DC-SIGN were observed between T-ALL and B-ALL patient cells. Also, no clear differences were seen between the overall binding of PB and BM cells.

Analysis of PB and BM cells from individual ALL patients resulted in a comparable binding to DC-SIGN in about 50% of the patients **(Fig. 2B)**. These patients showed a similar percentage of lymphoblasts in their PB and BM samples. The other ALL patients showed clear differences in binding to DC-SIGN and, except for T-ALL patient no.1, is correlated with the difference in percentage of blasts between PB and BM samples **(Fig. 2B)**. This indirectly indicates that DC-SIGN binds to the leukemic cells.

The binding results were also analysed according to developmental stage of the T- and B ALL cells, since differentiation of leukocytes can affect glycosylation of cell surface proteins [26]. Based on immunophenotyping, the T-ALL samples were subdivided into immature T-ALL, common T-ALL, and mature T-ALL [1,27]. Mature T-ALL showed an increased binding to DC-SIGN in comparison to immature T-ALL and common T-ALL (**Fig. 2C**, $p<0.05$). B-ALL was subdivided into pro-B-ALL, common B-ALL, and pre-B-ALL [1], but no clear differences in binding to DC-SIGN were detected (**Fig. 2C)**.

In conclusion, the increased binding of DC-SIGN to both B- and T-ALL cells indicates aberrant glycosylation confined to the leukemic cells and developmental stage in case of T-ALL.

Slightly increased binding of L-SIGN beads to ALL cells

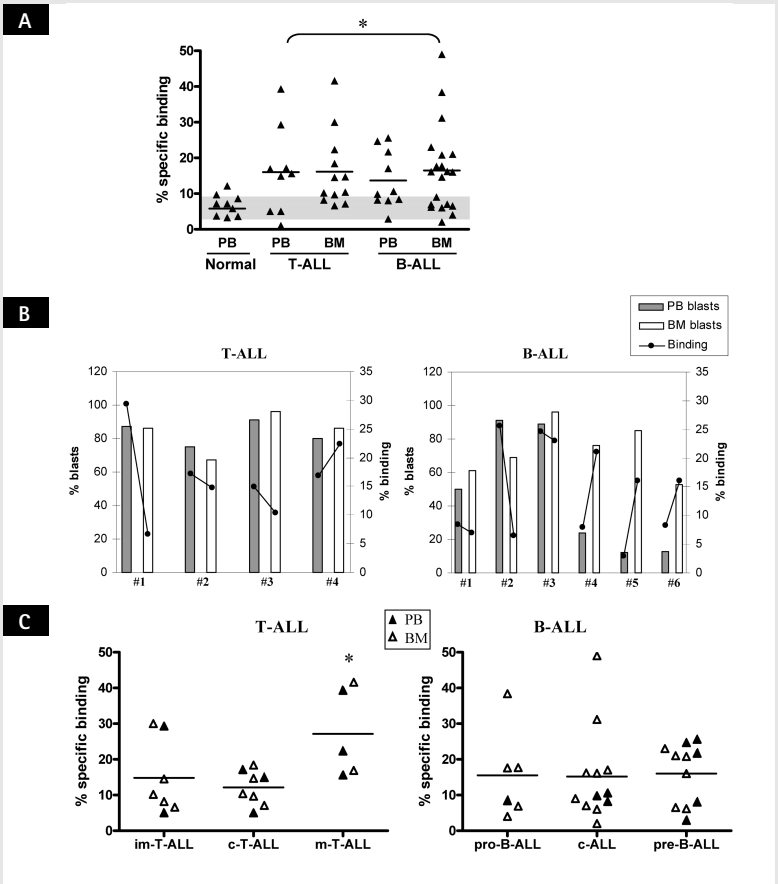
Next, the binding capacity of L-SIGN beads to PB and BM cells from T-ALL and B-ALL patients was tested. While mannan and EGTA were both equally potent in blocking the binding of DC-SIGN to ALL cells, this was not observed for L-SIGN. In 60% of the cases, EGTA was less potent than mannan in blocking the binding of L-SIGN beads to the cells **(Fig. 3A)**. Because of this partial blocking of EGTA, mannan was used to determine specific binding to L-SIGN in the various ALL patients tested **(Fig. 3B)**. BM, but not PB cells from B-ALL patients showed a significantly increased binding to L-SIGN beads when compared to PBL from healthy donors. PB and BM cells obtained from T-ALL patients both showed increased binding to L-SIGN beads. Similar to PBL from healthy donors, L-SIGN bound a higher percentage of ALL cells than DC-SIGN. Comparison of PB and BM cells from individual T-ALL patients showed differences in binding to L-SIGN despite a similar blast percentage **(Fig. 3C)**. In B-ALL patients the differences in binding to L-SIGN correlated with percentage of blasts.

The L-SIGN binding results were also studied for the developmental stages of T-ALL and B-ALL. This subdivision did not show a preference of L-SIGN in binding to a certain subtype **(Fig. 3D)**.

Thus, L-SIGN showed an increased binding to ALL cells, which was most pronounced for BM B-ALL cells. The degree of binding to L-SIGN is not dependent on the developmental stage of the ALL.

Figure 2

DC-SIGN BINDING TO ALL. (A) The binding of DC-SIGN-His coated beads to ALL cells derived from peripheral blood (PB) and/or bone marrow (BM) of leukaemia patients was determined. PBL from healthy donors was taken along for comparison. The cells were incubated for 30 min at 37°C with the DC-SIGN-His coated beads and subsequently analyzed by flow cytometry. Aspecific binding was determined by pretreatment of the DC-SIGN-His coated beads with 100 µg/ml mannan. Data are percentages of cells that displayed specific binding to the DC-SIGN-His coated beads. 95% Confidence interval of PBL from healthy donors (3-9% specific binding) represented by grey area. Significant difference from PBL from healthy donors as determined by unpaired one-tailed Student's t-test: * $p<0.05$. $p = 0.012$ from comparison of PBL healthy donors and PB T-ALL patients; $p = 0.005$ from comparison of PBL healthy donors and BM T-ALL patients; $p = 0.006$ from comparison of PBL healthy donors and PB B-ALL patients; $p = 0.006$ from comparison of PBL healthy donors and BM B-ALL patients. (B) Analysis of percentage of lymphoblasts in relation to DC-SIGN binding capacity. Percentage of lymphoblasts in ALL samples was determined morphologically and is depicted as bars. Percentage specific binding to DC-SIGN of the same ALL samples is depicted as connected dots. PB and BM cells from the same ALL patient is depicted. (C) Analysis of T- and B-ALL subtypes in relation to DC-SIGN binding capacity. The DC-SIGN binding data was categorized according to developmental stages of T-ALL; im-T-ALL (immature T-ALL), c-T-ALL (common T-ALL), and m-T-ALL (mature T-ALL) and B-ALL; pro-B-ALL, c-ALL (common B-ALL), and pre-B-ALL. Significant difference from immature T-ALL and common T-ALL according to ANOVA, followed by the Student Newman Keuls test: * $p<0.05$.



Characterization of DC-SIGN- and L-SIGN-binding ALL cells

The percentage of blasts did not correlate with the percentage of binding cells **(Fig. 2B** and **Fig. 3C)**. This indicates heterogeneity in glycosylation within the blast fraction. To determine which cell population binds to DC-SIGN and L-SIGN beads, triple labeling studies were performed. CD10 and CD34 were included to define the leukemic B-ALL population. Strikingly, the percentages of CD10 and CD34 positive and negative cells changed in the presence of DC-SIGN and L-SIGN beads when compared to control beads **(Fig. 4A)**. In the presence of DC-SIGN and L-SIGN beads, more $CD10^{-}CD34^{-}$ cells were detected whereas less $CD10^{+}CD34^{+}$ cells were found. This phenomenon is more pronounced in the presence of L-SIGN beads, which exhibit a higher binding to the cells (48%) than DC-SIGN (28%). Apparently, the beads inhibited the antibody binding as the cells are first incubated with the beads and then with the antibodies.

HSB2 cells were used to analyze this phenomenon by changing the sequence of incubation with beads and antibodies. To do so, one part of the HSB2 cells was incubated with DC-SIGN beads and subsequently with antibodies directed against CD5 and CD7 (**Fig. 4B,** manner 1). This incubation sequence resulted in 36.1% $CD5^{-}CD7^{-}$ and 3.9% $CD5^{+}CD7^{+}$ HSB2 cells. Strikingly, without DC-SIGN beads there are no $CD5^{-}CD7^{-}$ HSB2 cells and as much as 42.4% $CD5^{+}CD7^{+}$ HSB2 cells **(Fig. 4B)**. Changing the sequence of incubation by incubating HSB2 cells first with antibodies and then with DC-SIGN beads results in the presence of only 4.7% $CD5^{-}CD7^{-}$ cells and 21.2% $CD5^{+}CD7^{+}$ cells **(Fig. 4B**, manner 2). Therefore, this latter sequence of incubation comes closest to the CD5/CD7 staining pattern of HSB2 cells without any beads. However, this sequence of incubation caused a drastic decrease in binding of DC-SIGN to the HSB2 cells from 86% to 49% **(Fig. 4B)**.

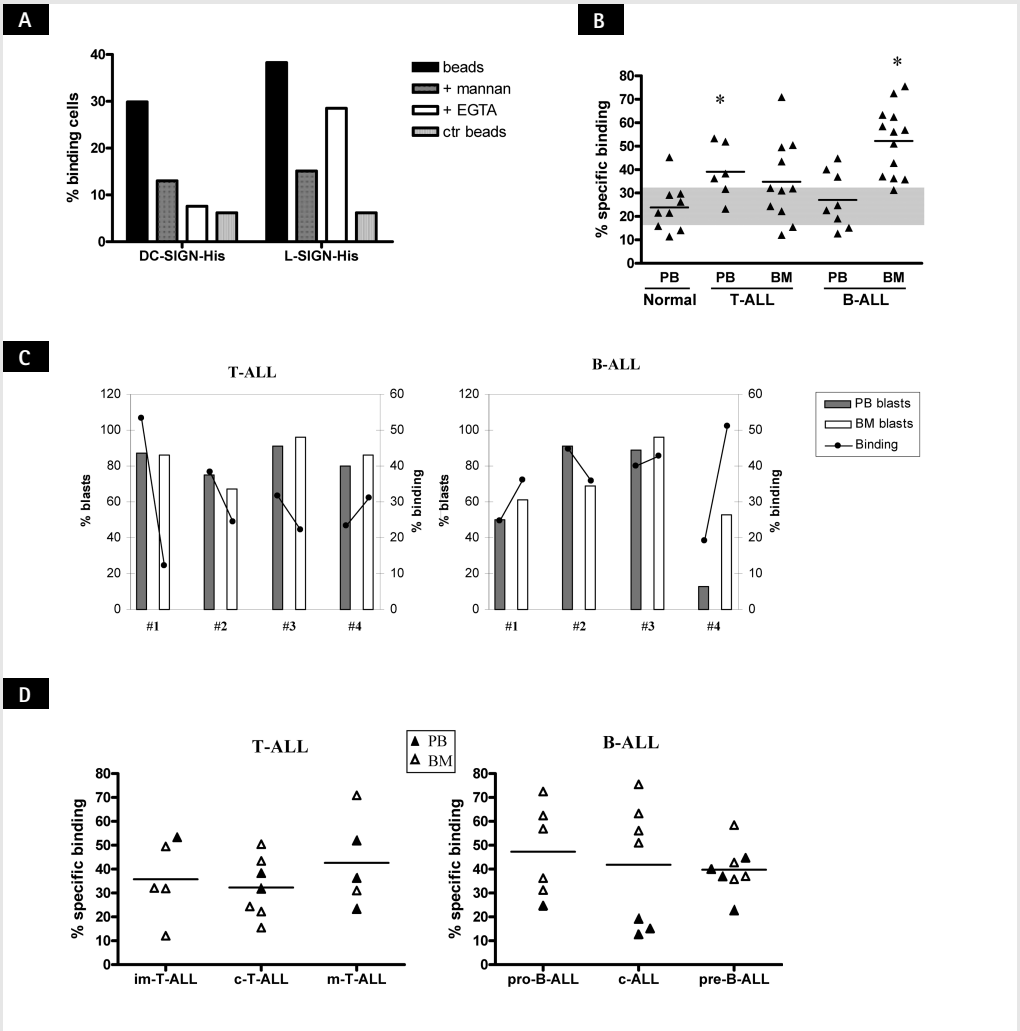
Overall, these data indicate that the beads and antibodies interfere in binding and therefore the triple labeling method can only be used to identify the binding population.

High CD15 (Lewis X) expression on B-ALL cells coincides with high binding to DC-SIGN

It was described that DC-SIGN can bind to colorectal cancer cells via Lewis X (CD15) [28]. This carbohydrate ligand is expressed at higher levels on colorectal cancer tissue compared to normal colon tissue. In haematopoietic tissues, CD15 expression is not associated with normal lymphoid cells, but it can be expressed in ALL [3,29]. Therefore, we correlated CD15 expression and binding to DC-SIGN beads on cells from the ALL patients. We used the 80H5 antibody as this antibody specifically recognizes unsialylated CD15, a ligand for DC-SIGN [30,31]. Analysis of CD24 expression on B-ALL cells was included as it has been suggested that this molecule might be a ligand for DC-SIGN and L-SIGN based on its specific surface glycosylation density [20]. Expression of CD15 and CD24 ranged from 0-80% and 4-98% positive cells in B-ALL respectively. Large differences were observed between the expression of these markers by individual patients. ALL cells from one patient showed 76.4% $CD15^{+}$ cells and 24.3% $CD24^{+}$ cells whereas cells from another patient showed 5.6% $CD15^{+}$ cells and as much as 94.9% $CD24^{+}$ cells **(Fig. 4C)**. A high CD15 expression on B-ALL cells (>10%) coincided with a high binding to DC-SIGN (>9%), but a substantial number of B-ALL patients with low levels of CD15 expression (<10%) also had a high binding to DC-SIGN **(Fig. 4D)**. This indicates that CD15 might be a ligand in some, but not in all B-ALL

Figure 3

L-SIGN BINDING TO ALL. (A) Binding of DC-SIGN-, L-SIGN- and control-His coated beads to PB cells isolated from a T-ALL patient. The cells were incubated for 30 min at 37°C with the His-construct coated beads and subsequently analyzed by flow cytometry. Aspecific binding was determined by pretreatment of the DC-SIGN- and L-SIGN-His constructs with 100 µg/ml mannan or 5 mM EGTA. Data are percentages of cells that displayed binding to the His-construct coated beads. (B) Analysis binding L-SIGN-His coated beads to PB and/or BM cells isolated from several ALL patients. PBL from healthy donors was taken along for comparison. Aspecific binding was determined by pretreatment of the L-SIGN-His coated beads with 100 µg/ml mannan. Data are percentages of cells that displayed specific binding to the L-SIGN-His coated beads. 95% Confidence interval of PBL from healthy donors (16-32% specific binding) represented by grey area. Significant difference from PBL from healthy donors as determined by unpaired one-tailed Student's t-test: * $p<0.05$. $p = 0.01$ from comparison of PBL healthy donors and PB T-ALL patients; $p<0.0001$ from comparison of PBL healthy donors and BM B-ALL patients. (C) Analysis of percentage of lymphoblasts in relation to L-SIGN binding capacity. Percentage of lymphoblasts in ALL samples was determined morphologically and is depicted as bars. Percentage specific binding to L-SIGN of the same ALL samples is depicted as connected dots. PB and BM cells from the same ALL patient is depicted. (D) Analysis of T- and B-ALL subtypes in relation to L-SIGN binding capacity. The L-SIGN binding data was separated according to developmental stages of T-ALL; im-T-ALL (immature T-ALL), c-T-ALL (common T-ALL), and m-T-ALL (mature T-ALL) and B-ALL; pro-B-ALL, c-ALL (common B-ALL), and pre-B-ALL.



patients. No relation was observed for CD24 expression (data not shown).
On T-ALL cells expression of CD15 was determined in combination with CD7 as the leukemic marker since CD24 is a marker for B cell lineage [32]. All T-ALL cells expressed rather low levels of CD15 (0-7%) **(Fig. 4C)**. The high binding to DC-SIGN beads as shown in **Fig. 2A** indicates the expression of other DC-SIGN ligands than CD15 in T-ALL.
Altogether, these data indicate that CD15 might be a ligand for DC-SIGN in some B-ALL patients, but due to negligible CD15 levels in other B- and T-ALL patients other ligands will play a role in binding to DC-SIGN.

High binding to BM cells is related to a better relapse-free-survival than high binding to PB cells

To investigate the prognostic significance of DC-SIGN and L-SIGN binding to ALL, we defined a low and high binding group based on the upper 95% confidence interval of the mean binding of PBL from healthy donors **(Fig. 2A, 3B)**. The 'low' binding group showed comparable binding as in healthy subjects (DC-SIGN less than 9%, L-SIGN less than 32%) while the 'high' binding group showed significant higher binding (DC-SIGN beyond 9%, L-SIGN beyond 32%). Further subdivision into PB and BM cells resulted in the following four groups: Low PB, low BM, high PB and high BM. In B-ALL, the high PB group showed the shortest relapse-free-survival, significantly different from the high BM group for both DC-SIGN and L-SIGN **(Fig. 5A)**. In T-ALL, the high PB group for DC-SIGN also showed a shorter relapse-free-survival although not significantly different from any of the other groups **(Fig. 5B)**. For L-SIGN the low PB T-ALL binders showed the shortest relapse-free-survival, but is not significantly different from any of the other groups **(Fig. 5A)**.
The four groups are heterogeneous with regard to gender, age, leukocyte count and cytogenetic background indicating aberrant glycosylation is not directly related to one of these prognostic markers **(Table 1 and 2)** [4,5].

Figure 4

CHARACTERIZATION OF DC-SIGN- AND L-SIGN-BINDING ALL CELLS. (A) CD10-FITC/CD34-PE dot plots of BM cells from a B-ALL patient binding to control-, DC-SIGN-, or L-SIGN-His coated beads. ALL cells were first incubated with the His-construct coated beads for 30 min at 37°C. Subsequently, the cells were labelled with CD10-FITC and CD34-PE for 20 min at RT and finally analysed by flow cytometry. Percentage of binding cells to the beads is depicted above the dot plots. Insets represent percentage positive cells for each quadrant. (B) HSB2 cells were incubated with DC-SIGN- and L-SIGN-His coated beads and antibodies in two different orders of sequence. In the first way (1), HSB2 were incubated with DC-SIGN- and L-SIGN-His coated beads for 30 min at 37°C and afterwards incubated with CD5-PE and CD7-FITC for 20 min at RT. In the second way (2), HSB2 cells were first incubated with CD5-PE and CD7-FITC for 20 min at RT and then with DC-SIGN- and L-SIGN-His coated beads for 30 min at 37°C. Finally, samples were analysed by flow cytometry. Percentage of binding cells to the beads is depicted above the dot plots. Insets represent percentage positive cells for each quadrant. (C) Expression profiles of CD15 and CD24 on B-ALL cells and CD15 and CD7 on T-ALL cells. Cells from several ALL patients were labelled with combinations of CD15-PE and CD24-FITC or CD15-PE and CD7-FITC for 20 min at RT. Samples were analysed by flow cytometry. Percentage of binding cells to the beads is depicted above the dot plots. Insets represent percentage positive cells for each quadrant. (D) Analysis of CD15 expression on B-ALL cells in relation to DC-SIGN binding capacity. Percentage of CD15 positive cells on B-ALL cells as determined by 80H5 antibody was plotted against specific binding to DC-SIGN beads (determined with mannan).

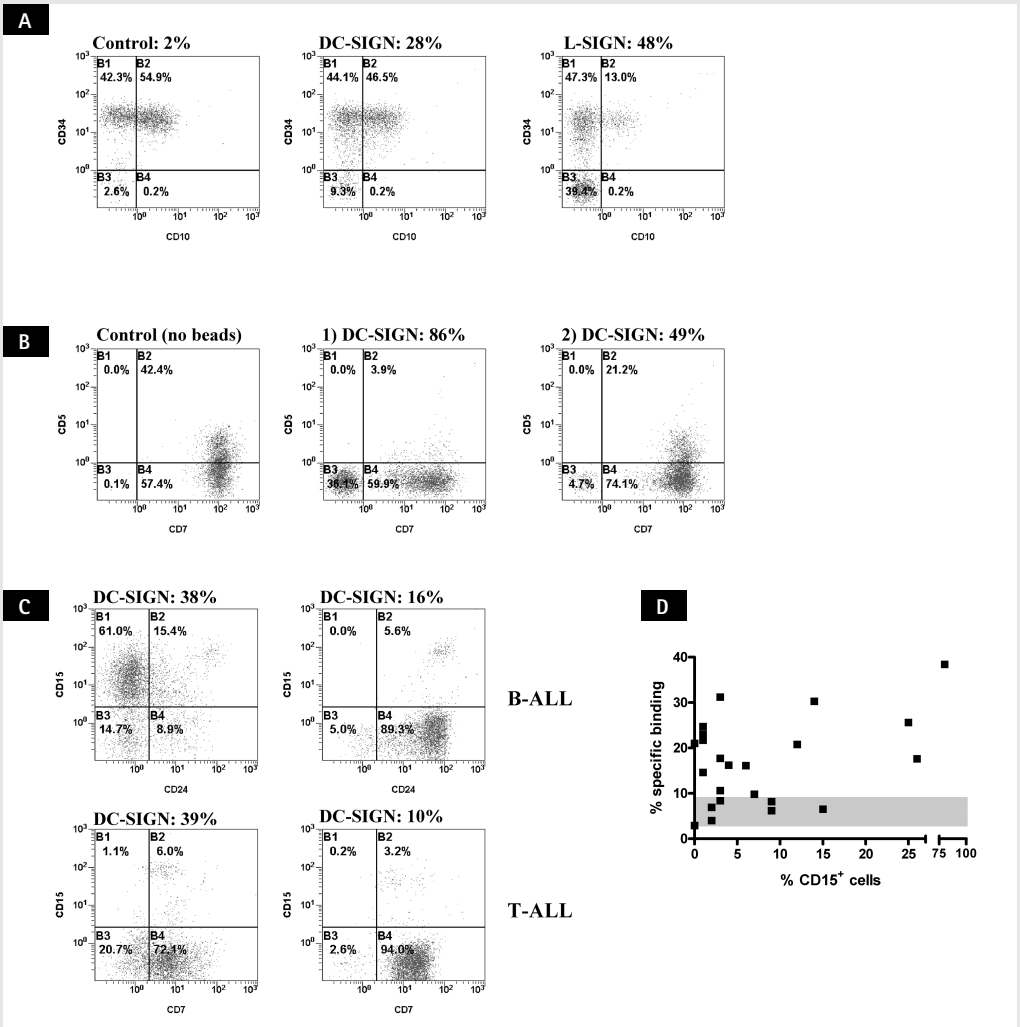


Figure 5

HIGH BINDING TO B-ALL PB CELLS IS CORRELATED WITH POOR DISEASE OUTCOME. Analysis of relapse-free-survival of B-ALL (A) and T-ALL (B) patients with regard to DC-SIGN and L-SIGN binding capacity. Binding data were categorized into Low (DC-SIGN <9%, L-SIGN <32%) and High (DC-SIGN >9%, L-SIGN >32%) PB and BM binding groups based on the upper 95% confidence interval of the mean binding of PBL from healthy donors **(Fig. 2A-3B)**. Kaplan-Meier curves were made by SPSS software. Significant different from high BM group as determined by log-rank test: * p=0.0316 (DC-SIGN), * p=0.0284 (L-SIGN).

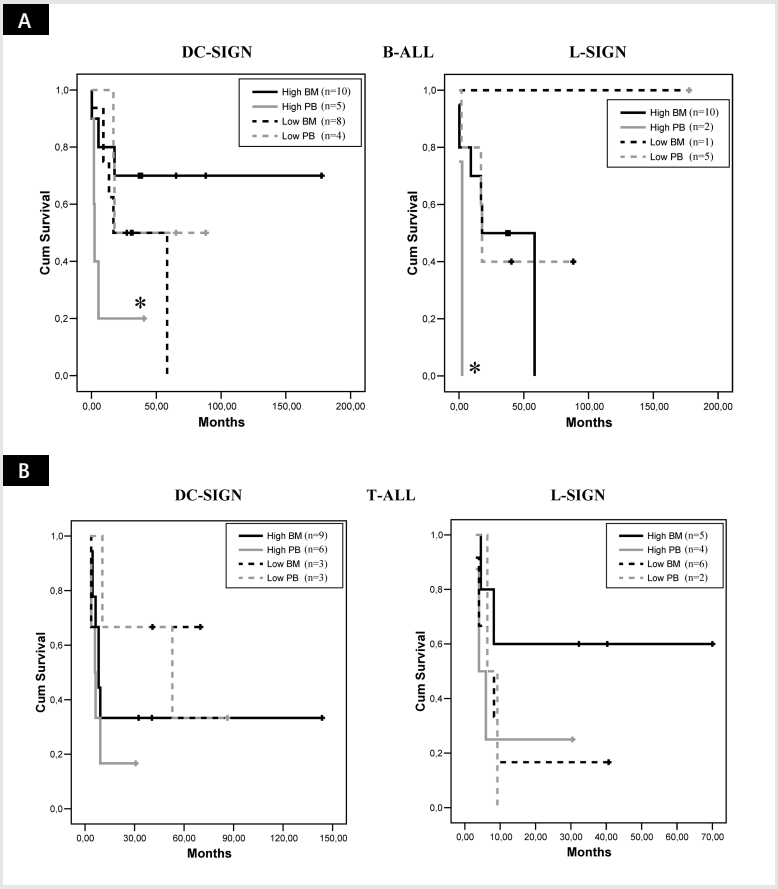


Table 1

CHARACTERISTICS OF B-ALL STUDY POPULATION. Characteristics of the B-ALL patients tested in this study. (Cyto)genetic data were obtained by routine cytogenetics and interphase FISH. Data were categorized according to presence of 0 deviations (Normal), 1-2 deviations, more than 2 deviations (Complex), more than 46 chromosomes per cell (Hyperdiploid), breakpoint cluster region-Abelson translocation (BCR-ABL1), mixed-lineage-leukaemia translocation (MLL), and ETS variant gene 6-runt related transcription factor 1 (ETV6-RUNX1). RFS: relapse-free-survival; CR: complete remission; D: dead; F: female; M: male; NA: not available.

Patient	Group DC-SIGN	Group L-SIGN	Status	RFS (months)	Age (years)	Sex	Cytogenetics	Blasts (%)	Leukocytes ($\times 10^9$ cells/L)
1	Low PB High BM	-	CR	65	4	M	ETV6-RUNX1	24 76	9
2	Low PB Low BM	Low PB High BM	D	17	18	F	MLL-rearrangement	50 61	38
3	Low PB High BM	Low PB/-	CR	88	7	F	ETV6-RUNX1	12 85	3 102
4	Low PB High BM	Low PB High BM	D	18	43	M	BCR-ABL1	13 53	6.2
5	High PB	Low PB	CR	41	45	F	1-2 Deviations	NA	NA
6	High PB	Low PB	D	2	64	F	Complex/ MLL-rearrangement	72	76
7	High PB	High PB	D	2	20	F	Normal	13	5.2
8	High PB Low BM	High PB High BM	D	0.1	45	M	MLL-rearrangement	91 69	251
9	High PB High BM	-	D	5	6	M	BCR-ABL1	92 NA	285
10	Low BM	-	D	14	13	F	BCR-ABL1	91	31.2
11	Low BM	-	CR	27	4	F	Normal	95	15.4
12	Low BM	-	CR	32	6	M	1-2 Deviations	85	1.6
13	Low BM	-	CR	31	2	M	Hyperdiploid	96	24
14	Low BM	High BM	D	58	35	F	Hyperdiploid	88	8.2
15	Low BM	High BM	D	9	3	F	Normal	56	3.5
16	High BM	High BM	CR	39	17	M	Hyperdiploid	80	31
17	High BM	High BM	CR	37	2	M	MLL-rearrangement	91	139
18	High BM	High BM	CR	39	5	F	ETV6-RUNX1	91	1.4
19	High BM	High BM	D	0.3	49	M	1-2 Deviations	97	12.4
20	High BM	High BM	CR	38	12	M	ETV6-RUNX1	97	6
21	High BM	Low BM	CR	178	51	F	Normal	65	200

Table 2

CHARACTERISTICS OF T-ALL STUDY POPULATION. Characteristics of the T-ALL patients tested in this study. Cytogenetic data were categorized according to presence of 0 deviations (Normal), 1-2 deviations, more than 2 deviations (Complex), more than 46 chromosomes per cell (Hyperdiploid), and mixed-lineage-leukaemia translocation (MLL). RFS: relapse-free-survival; CR: complete remission; D: dead; F: female; M: male; NA: not available.

Patient	Group DC-SIGN	Group L-SIGN	Status	RFS (months)	Age (years)	Sex	Cytogenetics	Blasts (%)	Leukocytes ($\times 10^9$ cells/L)
1	Low PB	-	CR	86	35	F	Complex	42	10.5
2	Low PB	-	D	53	34	F	1-2 Deviations	29	53
3	Low PB	-	D	11	41	M	Complex	59	18.5
4	High PB High BM	Low PB Low BM	D	9	31	M	1-2 Deviations	91 96	188
5	High PB High BM	Low PB Low BM	D	6	29	M	Normal	80 86	147
6	High PB Low BM	High PB Low BM	D	4	48	F	Normal	87 87	122
7	High PB High BM	High PB Low BM	D	3	17	M	1-2 Deviations	75 67	800
8	High PB	High PB	CR	30	31	M	1-2 Deviations	19	13.3
9	High PB	High PB	D	6	16	M	1-2 Deviations	NA	NA
10	Low BM	Low BM	CR	41	11	M	Hyperdiploid	88	42
11	Low BM	High BM	CR	70	12	F	MLL-rearrangement	85	41.3
12	High BM	Low BM	D	8	16	F	NA	77	280
13	High BM	High BM	D	8	20	M	1-2 Deviations	79	28.1
14	High BM	High BM	D	5	14	M	Normal	65	266
15	High BM	High BM	CR	32	6	M	Normal	85	25
16	High BM	High BM	CR	40	5	M	1-2 Deviations	95	37
17	High BM	-	CR	143	22	M	Complex	94	24

Discussion

In this study the binding capacity of recombinant DC-SIGN and L-SIGN to cells from ALL patients was analyzed. Most ALL cells showed an increased binding to these two C–type lectins when compared to PBL from healthy donors. This indicates that ALL cells have an altered glycosylation pattern resulting in increased expression of DC-SIGN and L-SIGN ligands. Overall, T-ALL and B-ALL cells showed no difference in DC-SIGN binding. PB and BM cells from the same ALL patients demonstrated a similar binding. In contrast, L-SIGN showed a higher binding to BM cells isolated from B-ALL patients than leukemic cells collected from peripheral blood. The lower binding of L-SIGN by PB cells from B-ALL patients might be explained by a lower percentage of blasts as depicted in **Fig. 3C** patient 4 and **Table 1**. The difference between B-ALL and T-ALL patients in binding to L-SIGN implies an increased expression of L-SIGN ligands on B-ALL cells.

Comparison of PB and BM from an individual ALL patient showed similar binding to DC-SIGN or L-SIGN when corrected for the percentage of blast cells **(Fig. 2B, 3C)**. However, in some ALL patients (especially T-ALL) this was not found suggesting heterogeneity in the levels of aberrant glycosylation. Possibly, the location (BM vs. blood) plays a role in the degree of aberrant glycosylation [33].

Separation of T- and B-ALL into subtypes according to chronological development did not reveal a significant distinction in binding to DC-SIGN and L-SIGN beads except for mature T-ALL. This subgroup showed an increased binding to DC-SIGN and also slightly to L-SIGN when compared to immature and common T-ALL. The high binding might be caused by a higher extend of specific glycosylation in this subtype.

Due to sterical hindrance, the binding populations could not be identified unambiguously. Besides sterical hindrance, possibly some quenching of the antibodies does take place since incubating first with antibodies and then with beads still showed some $CD5^{-}CD7^{-}$ HSB2 cells.

The higher binding capacity of L-SIGN compared with DC-SIGN in ALL cells and PBL from healthy donors suggests the presence of additional ligands for L-SIGN. This is unexpected as previous studies indicate DC-SIGN has a broader binding specificity than L-SIGN like CD15 that might be a DC-SIGN ligand in some B-ALL patients [14,19]. The poor blocking potential of EGTA to abrogate binding of L-SIGN to several ALL samples and less frequently also to PBL indicates that part of the binding is Ca^{2+}-independent. This is quite unusual for a C-type lectin [9]. Interestingly, the C-type lectin Dectin-1 recognizes an as yet unidentified ligand on D10 T cells in an unconventional C-type lectin way as well [34]. To examine this in more detail, future studies are required to identify the ligands for both DC-SIGN and L-SIGN expressed on the ALL cells.

The physiological significance of DC-SIGN and L-SIGN interacting with ALL cells remains to be determined, but the poorer survival with increased binding to B-ALL PB cells suggests immune escape of the ALL cells. Escape from immune surveillance is not uncommon to ALL and although several factors are suggested to play a role like T cell defects, lack of costimulation, and immunosuppressive cytokines the mechanism is still unknown [35,36]. We suggest that once the leukemic cells enter the blood circulation, and are then

considered to be PB cells, they can interact with DC-SIGN and L-SIGN positive cells. These cells include DC on which DC-SIGN is expressed and are frequently postulated in immune escape of tumor cells [28,37,38]. DC can induce tolerance in a steady-state situation when no other antigen-sensing receptors like Toll-like receptors are triggered [39]. This is an important mechanism to prevent damage against endogenous tissues but for tumor cells this can be a way to escape from immune surveillance. Furthermore, interaction of ALL cells with DC-SIGN on DC may change the direction of the immune response like the pathogens *Mycobacterium tuberculosis* and *Helicobacter pylori* do by binding to DC-SIGN [40,41]. In this way those pathogens and possibly also leukemic cells can escape the immune system. Additionally, LSEC express both DC-SIGN and L-SIGN and can induce T cell tolerance [17,42]. This tolerance induction is beneficial for innocuous antigens in the circulation, but harmful when altered cells like ALL are tolerized. Future studies are necessary to test these concepts and may provide new therapeutic tools to treat ALL. Moreover, the degree of binding of DC-SIGN- and L-SIGN-beads to B-ALL PB cells may be a useful prognostic tool if tested in larger series of patients since the clinical significance within this small group of patients has to be interpreted with caution.

For T-ALL no significant differences could be detected in the survival analysis **(Fig. 5B)**. However like B-ALL, the high binding PB group to DC-SIGN also has a relatively poor survival in comparison to the other groups. This may suggest similar immune escape mechanisms. With L-SIGN however, the low PB T-ALL group has a lower survival than the high PB T-ALL group which indicates that survival of T-ALL is not dependent on binding to L-SIGN positive cells.

In conclusion, this study demonstrates a generally increased binding of ALL cells to DC-SIGN and L-SIGN indicating aberrant glycosylation on these leukemic cells. Increased binding to PB cells from B-ALL patients is associated with a poor survival and therefore seems to be beneficial for the leukemic cells. This may involve a tolerating mechanism by interacting with DC-SIGN and L-SIGN positive cells.

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Chapter 7

Effective induction of naïve and recall T cell responses by targeting antigen to human dendritic cells via a humanized anti-DC-SIGN antibody

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Abstract

Current dendritic cell (DC)-based vaccines are based on *ex vivo* generated autologous DC loaded with antigen prior to readministration into patients. A more direct and less laborious strategy is to target antigens to DC *in vivo* via specific surface receptors. Therefore, we developed a humanized antibody, hD1V1G2/G4 (hD1), directed against the C-type lectin DC-SIGN, to explore its capacity to serve as a target receptor for vaccination purposes. hD1 was cross-linked to a model antigen, keyhole limpet hemocyanin (KLH). We observed that the chimeric antibody-protein complex (hD1-KLH) bound specifically to DC-SIGN, was rapidly internalized and translocated to the lysosomal compartment. To determine the targeting efficiency of hD1-KLH, monocyte-derived DC and peripheral blood lymphocytes (PBL) were obtained from patients who had previously been vaccinated with KLH-pulsed DC. Autologous DC pulsed with hD1-KLH induced proliferation of patient PBL at a 100-fold lower concentration than KLH-pulsed DC. In addition, hD1-KLH-targeted DC induced proliferation of naïve T cells recognizing KLH epitopes in the context of MHC class I and II. We conclude that antibody-mediated targeting of antigen to DC via DC-SIGN effectively induces antigen-specific naïve- as well as recall T cell responses. This identifies DC-SIGN as a promising target molecule for DC-based vaccination strategies.

Introduction

Dendritic cells (DC) are professional antigen-presenting cells (APCs) that play a key role in regulating antigen-specific immunity. DC capture antigens, process them into peptides and present these to T cells [1]. The interaction between DC and T cell controls the type and magnitude of the resulting immune response. Recently, preclinical and clinical studies have exploited DC in an attempt to improve vaccine efficacy [2]. Most of these studies involve *ex vivo* antigen loading of autologous monocyte-derived DC that are re-administrated to the patient, a laborious and costly procedure. A more direct strategy involves targeting of antigens specifically to antigen uptake receptors on the DC *in vivo*. Potential candidate receptors highly expressed by DC include Fc receptors [3-5] and members of the C-type lectin family [6,7]. Whereas Fc receptors are expressed by many different cell types, the expression of some members of the C-type lectin family are more DC-restricted [8].

C-type lectins bind sugar residues in a calcium-dependent manner via a highly conserved carbohydrate recognition domain. C-type lectin receptors expressed by DC are implicated in immunoregulatory processes, such as antigen capture, DC trafficking and DC-T cell interactions [9]. Based on the location of the amino (N) terminus, two types of membrane-bound C-type lectins can be distinguished on DC. Type I C-type lectins have their N terminus located outside, while type II C-type lectins have their N terminus located inside the cell. Several studies have been conducted on antigen targeting to C-type lectin receptors for vaccination purposes, mainly focusing on the type I C-type lectins mannose receptor (MR) [10] and DEC-205 [11-13]. Vaccines based on natural MR ligands have been shown to effectively induce humoral and cellular responses [14]. However, these ligands lack specificity for the MR, and may target multiple lectins with overlapping binding specificities, including soluble lectins and lectin receptors expressed by cells that are not specialized in antigen presentation. More specific receptor targeting can be obtained by the use of antibodies directed against specific C-type lectins, a strategy that has been successfully applied in MR [15,16] and DEC-205 [17-19] targeting studies. Antibody-mediated targeting of antigen to the MR on human DC results in antigen presentation and activation of naïve T cells *in vitro* [20,21]. Moreover, *in vivo* studies on antibody-mediated targeting of DEC-205 in mice demonstrate presentation of the antigen to naïve $CD4^+$ and $CD8^+$ T cells [22,23].

DC-specific ICAM-3 grabbing non-integrin (DC-SIGN) represents a member of the type II C-type lectin family. We have previously demonstrated that DC-SIGN is an endocytic receptor mediating antigen presentation [24]. A major advantage of targeting DC-SIGN over other C-type lectin receptors is its expression pattern. In humans, DC-SIGN expression is restricted to professional APCs and expression levels are high. Human DC-SIGN is abundantly expressed by DC residing in lymphoid tissues and at mucosal surfaces, dermal DC, and by specialized macrophages in placenta and lung [25,26].

Targeting constructs that are to be used in humans should consist of antibodies that do not elicit immune responses directed against the antibody itself. Recent developments in antibody engineering provide the tools for the production of either humanized or human antibodies [27]. Undesired interactions between the targeting antibody and Fc receptors can be avoided by use of single chain Fv constructs or composite IgG molecules [28], thus enhancing targeting specificity.

Here we evaluate the effectiveness of targeting antigen to human DC via DC-SIGN. For targeting purposes, keyhole limpet hemocyanin (KLH), a large globular protein containing a large array of immunogenic epitopes, was chosen as a model antigen. KLH is widely used in clinical DC-based vaccination trials for immunomonitoring purposes, and is thought to stimulate cytotoxic T cell responses by recruiting bystander T cell help [29]. KLH was chemically cross-linked to a humanized anti-DC-SIGN IgG2/IgG4 composite antibody (hD1), resulting in the chimeric hD1-KLH protein. The results demonstrate that hD1-KLH was capable of inducing T cell responses at a 100-fold lower concentration than KLH alone.

Materials and Methods

Antibodies and reagents

The following antibodies were used: AZN-D1 (IgG1, mouse anti-human DC-SIGN) [30], AZN-L19 (IgG1, mouse anti-human CD18) [31], W6/32 (IgG2a, mouse anti HLA-A, -B, -C; ATCC, Manassas, VA), IVA-12 (IgG1, mouse anti HLA-DR, -DP, -DQ; ATCC), mouse IgG1 isotype (R&D systems, Abingdon, UK), total mouse IgG (Jackson ImmunoResearch; Brunschwig Chemie B.V., Amsterdam, The Netherlands), Alexa Fluor 647-labeled goat anti-human IgG (Molecular Probes, Leiden, The Netherlands) and Alexa Fluor 647-labeled goat anti-mouse IgG1 (Molecular Probes). Endotoxin-free KLH was purchased from Calbiochem (La Jolla, CA).

Recombinant antibodies

The humanized anti-human DC-SIGN antibody hD1V1G2/G4 (hD1) was generated by complementarity determining region (CDR)-grafting of AZN-D1 hypervariable domains into human framework regions. The humanized variable heavy and variable light regions were then genetically fused with a human hybrid IgG2/IgG4 constant domain [32] and a human kappa chain constant domain, respectively. This construct was cloned into a mammalian expression vector and the final construct transfected into NSO cells. Stable transfectants were obtained using glutamate synthetase (GS) selection (Lonza Biologics, Portsmouth, NH). Supernatants containing hD1 were purified over a Protein A column. An isotype control antibody, h5G1.1-mAb (5G1.1; Eculizamab; Alexion Pharmaceuticals, Inc.) containing the same IgG2/IgG4 constant region, is specific for the human terminal complement protein C5 [33].

Generation of hD1-KLH

The chemical cross-linker sulfosuccinimidyl 4-(N-maleimidomethyl)-cyclohexane-1-carboxylate (sSMCC; Pierce, Rockford, IL) was conjugated to KLH according to the manufacturer's protocol. Protected sulfhydryl groups were introduced to the hD1 antibody with N-succinimidyl-S-acetylthiopropionate (SATP; Pierce), and were reduced with hydroxylamine hydrochloride (Pierce) using the manufacturer's protocol. Subsequently, hD1 was added to sSMCC-treated KLH in phosphate buffered saline (pH 7.4) and allowed to react for 16 h at 4°C. Unbound sites were alkylated by adding iodoacetamide (Sigma-Aldrich, St. Louis, MO) to a final concentration of 25 mM, followed by 30 min incubation at room temperature. The protein mixture was loaded onto a Superose 6 column (24 ml bed volume; Amersham Pharmacia Biotech, Uppsala, Sweden), and fractions were collected and analyzed by SDS-PAGE. Fractions containing hD1-KLH were pooled and fractions containing free hD1 were discarded. The efficiency of the cross-linking reaction was estimated by comparing the amount of hD1 relative to KLH before the reaction to the hD1 to hD1-KLH ratio after cross-linking. We calculated that, on average, each KLH molecule had reacted with 10 hD1 molecules (data not shown). Endotoxin levels of the pooled hD1-KLH fractions were below detection levels (< 0.04 pg/µg protein) in the QCL-1000 *Limulus* amebocyte lysate assay (BioWhittaker, Walkersville, MD).

Monocyte-derived DC and PBL

Peripheral blood mononuclear cells (PBMC) were obtained from buffy coats of healthy individuals and were purified using Ficoll density centrifugation. PBL and immature DC (iDC) were obtained from PBMC as reported elsewhere [34]. In brief, PBMC were allowed to adhere for 1 h at 37°C. Non-adherent cells (PBL) were gently removed, washed and cryopreserved. The adherent monocytes were cultured in the presence of IL-4 and GM-CSF (500 and 800 U/ml, respectively; Schering-Plough International, Kenilworth, NJ) for 6 days to obtain immature DC. Mature DC (mDC) were obtained by culturing iDC in the presence of 2 µg/ml lipopolysaccharide (LPS) for 24h. DC were cryopreserved until use. Unless indicated otherwise, cells were cultured in X-VIVO 15 medium (Cambrex, Verviers, Belgium) supplemented with 2% human serum.

Tritiated thymidine incorporation assays

Tritriated thymidine (1 µCi [0.037 Mbq]/well; MP Biomedicals, Amsterdam, The Netherlands) was added to the cell cultures. Tritiated thymidine incorporation was measured after 16 h in a ß-scintillation counter. Proliferation indices higher than 2 were considered positive.

Clinical vaccination protocol

PBL and monocyte-derived DC were isolated from melanoma patients participating in a clinical vaccination trial, as described by de Vries *et al.* [35]. Patients were determined to have stage IV disease according to the American Joint Committee on Cancer (AJCC) criteria [36]. The study was approved by the institutional review board (Radboud University Nijmegen Medical Centre, Commissie Mensgebonden Onderzoek). Informed consent was provided according to the Declaration of Helsinki. The vaccination protocol consisted of two parts. In the first part, antigen-pulsed mDC were administered intravenously and intradermally, 3 times at bi-weekly intervals. In the second part, patients received 3 monthly intradermal vaccinations with peptides alone (100 µg) and KLH (2 µg). Patients who remained free of disease progression after the first vaccination cycle were eligible for maintenance cycles at six-month intervals, each consisting of 3 bi-weekly intranodal vaccinations in a clinically tumor-free, lymph node region under ultrasound guidance with mDC alternately pulsed with wild type or modified gp100 peptides [37], tyrosinase peptides and KLH. Clinical grade DC for vaccination purposes were generated from peripheral blood mononuclear cells (PBMC) as described previously [38].

Humoral responses to KLH

Humoral responses to KLH were determined by enzyme-linked immunosorbent assays (ELISA) as described by Holtl *et al.* [39]. Briefly, 96-well plates were coated overnight at 4°C with the protein KLH (25 µg/ml) in phosphate-buffered saline (PBS; 0.1 ml/well). Subsequently, plates were incubated with serial dilutions of patient serum, obtained before and during the third maintenance cycle, for 1 h at room temperature. After extensive washing, human IgG-specific antibody labeled with horseradish peroxidase was allowed to bind for 1 h at room temperature. Peroxidase activity was revealed using 3,3' 5,5' tetramethyl-benzidine as substrate and measured in a microtiter plate reader at 450 nm. A signal detected at ≥ 1:400 dilution of serum was considered positive.

Cellular responses to KLH

Cryopreserved PBMC, isolated from peripheral blood samples taken from the patients, were thawed, washed and plated at $1x10^5$ PBMC per well of a 96-well tissue culture microplate either in the presence or absence of 10 µg/ml KLH. After 4 days of culture, a tritiated thymidine incorporation assay was performed.

Binding and internalization assays

Binding of hD1 and hD1-KLH to iDC was assessed by immunofluorescence and flow cytometry. iDC were incubated with or without 10 µg/ml of hD1 or hD1-KLH. In some experiments, DC-SIGN was blocked by pretreating iDC with 100 µg/ml AZN-D1. After a 1 h incubation at 4°C, cells were washed and incubated with Alexa Fluor 647-labeled anti-human IgG antibody. Cells were analyzed on a FACScalibur flow cytometer using CellQuest software (BD Biosciences, San Jose, CA).

Internalization of hD1 by iDC was determined by flow cytometry as described previously [40]. Briefly, iDC were incubated with 10 µg/ml hD1, AZN-D1, AZN-L19 or mouse IgG1 and 5G1.1 isotype control antibodies at 4 °C for 30 min, washed, and incubated for 0, 15, 30 or 45 min at 37°C. Subsequently, some of the cells were fixed, while others were fixed and permeabilized in PBS/0.1% (vol/wt) saponin (Sigma-Aldrich) before addition of the Alexa Fluor 647-labeled anti-human IgG secondary antibody. The amount of internalized antibody was calculated by subtracting the mean fluorescence in fixed cells (surface-bound) from that recorded with fixed and permeabilized cells (internalized and surface-bound) at the various time points.

Internalization of hD1 and hD1-KLH was confirmed by confocal laser scanning microscopy (CLSM). iDC were incubated with 10 µg/ml hD1, hD1-KLH, AZN-D1 or isotype control 5G1.1 and mouse IgG1 antibodies for 1 h at 37°C. Cells were fixed on poly-L-lysine coated glass slides, followed by intracellular staining with Alexa Fluor 647-labeled secondary antibodies. Cells were imaged with a Bio-Rad MRC 1024 confocal system operating on a Nikon Optiphot microscope and a Nikon 60x planApo 1.4 oil immersion lens (BioRad, Hercules, CA). Pictures were analyzed with Bio-Rad Lasersharp 2000 and Adobe Photoshop 7.0 (Adobe Systems, Mountain View, CA) software.

KLH binding and internalization by iDC was assessed by direct labeling of KLH using the Alexa Fluor 488 labeling kit (Molecular Probes). iDC were incubated with 10 µg/ml labeled KLH for 1 h at either 4 or 37°C. Subsequently, cells were washed and analyzed by flow cytometry.

Live imaging of hD1-KLH and KLH uptake by iDC

iDC were labeled with LysoTracker Red (Molecular Probes) in PBS for 10 min at room temperature. Subsequently, cells were transferred to RPMI 1640 without phenol red (Gibco; Life Technologies, Breda, The Netherlands) supplemented with 1% human serum. Labeled cells were analyzed at 37°C with a Zeiss LSM 510 microscope equipped with a type S heated stage CO_2 controller and PlanApochromatic 63x 1.4 oil immersion DIC lens (Carl Zeiss GmbH, Jena, Germany). KLH and hD1-KLH, directly labeled with the Alexa Fluor 488 labeling kit (Molecular Probes), were added to the medium at 10 µg/ml. Cells were imaged using Zeiss LSM Image Browser version 3.2 (Carl Zeiss) and processed with Image J version 1.32j software (National Institutes of Health, http://rsb.info.nih.gov/ij).

Targeting experiments

iDC were incubated with hD1, hD1-KLH or KLH for 1 h at 4 or 37°C. Where indicated, iDC were matured with LPS. Subsequently, DC were washed and cocultured with KLH-responsive PBL (ratio 1:10) at 37°C. In some experiments, iDC were matured with LPS before addition of PBL. After 16 h of coculture, IL-2, IL-4, IL-5, IL-10, TNF-α, and IFN-γ were measured by cytometric bead array (CBA) (Th1/Th2 Cytokine CBA 1; BD Pharmingen, San Diego, CA) according to the manufacturer's instructions. After 4 days of coculture, a tritiated thymidine incorporation assay was performed.

Presentation of KLH epitopes over time

iDC were incubated with 5 µg/ml KLH or 5 µg/ml hD1-KLH for 1 h at 37°C. Following washing, KLH-responsive PBL were added to the iDC either on the same day, or 2 or 4 days later. Four days after addition of the PBL, proliferative responses were determined in a tritiated thymidine incorporation assay.

Activation and expansion of naïve T cells

Experiments were performed essentially as described previously [41]. Briefly, PBL from a healthy donor were used as a source of T cells. Autologous DC were incubated with 10 µg/ml hD1-KLH for 1 h at 37°C, washed, and matured. Targeted mDC were incubated with PBL (ratio 1:10) in the presence of IL-7 (10 ng/ml, day 0), followed by addition of IL-10 (10 ng/ml) on day 1 and IL-2 (20 U/ml) on day 2. IL-2 was added to the culture every 3 to 4 days. PBL were restimulated each week. Restimulations were performed with iDC treated with 5 µg/ml hD1-KLH after 1 week, and with iDC treated with 2.5 µg/ml hD1-KLH after 2, 3 and 4 weeks. Finally, PBL were harvested and cocultured with autologous mDC that had been pulsed with KLH, as described in "Targeting experiments". Proliferative responses were determined by tritiated thymidine incorporation assays.

Results

Humanized anti-DC-SIGN antibody hD1 is internalized by iDC

The CDRs of the mouse anti-human DC-SIGN antibody AZN-D1 were grafted onto a human IgG2/IgG4 composite antibody to generate a humanized antibody for DC-SIGN targeting, hD1. We have previously shown that the human hybrid IgG2/IgG4 constant domain prevents antibodies from binding to Fc receptors [42]. The binding affinity of hD1 for DC-SIGN was similar to that of AZN-D1 as determined by surface plasmon resonance (3.7 ± 0.7 and 3.8 ± 1.1, respectively, data not shown).

Flow cytometric analysis revealed specific binding of hD1 to DC-SIGN on iDC, as preincubation of iDC with AZN-D1 efficiently reduced binding **(Fig. 1A)**. Furthermore, hD1 bound to DC-SIGN expressing K562 cells after transfection with DC-SIGN cDNA, whereas it did not bind to untransfected K562 cells

Figure 1

BINDING OF HD1 TO DC-SIGN AND INTERNALIZATION BY DC. (A) iDC were treated with 10 µg/ml hD1 (gray shaded curve), 100 µg/ml AZN-D1 (open dotted curve), or pretreated with 100 µg/ml AZN-D1 followed by 10 µg/ml hD1 incubation (open solid curve), followed by incubation with an Alexa Fluor 647-labeled goat anti-human IgG antibody. Binding of hD1 was analyzed by flow cytometry. (B) iDC were incubated with AZN-D1 (•), hD1 (o) or AZN-L19 (□) at 4°C for 1 h, and transferred to 37°C. Cells were fixed at various time points, and stained with an Alexa Fluor-labeled secondary antibodies with or without prior permeabilization. The mean fluorescence was determined by flow cytometric analysis and the amount of internalized antibody was plotted as a percentage of the amount of total cell-associated antibody. Data represent experiments performed in triplicate ± SD. (C) Internalization of hD1 was confirmed by CLSM. iDC were incubated with hD1, AZN-D1, or their isotype controls 5G1.1 and mouse IgG1 (mIgG) for 1 h at 37°C. Cells were stained with Alexa Fluor 647-labeled secondary antibodies (blue), followed by microscopic analysis. The image represents the middle focal plane of the DC, with iris set at 2 nm. Original magnification, × 600.

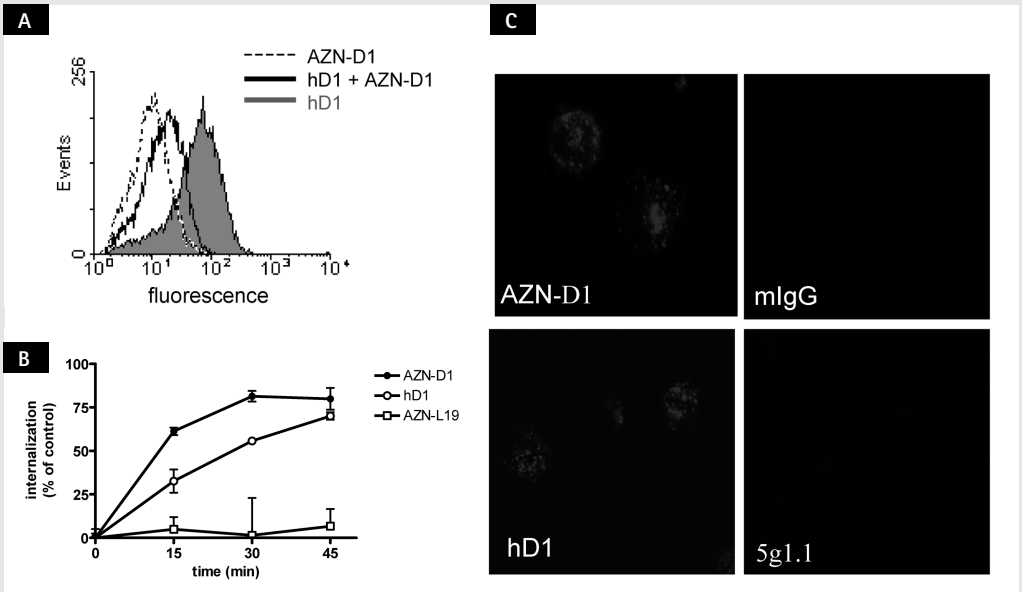
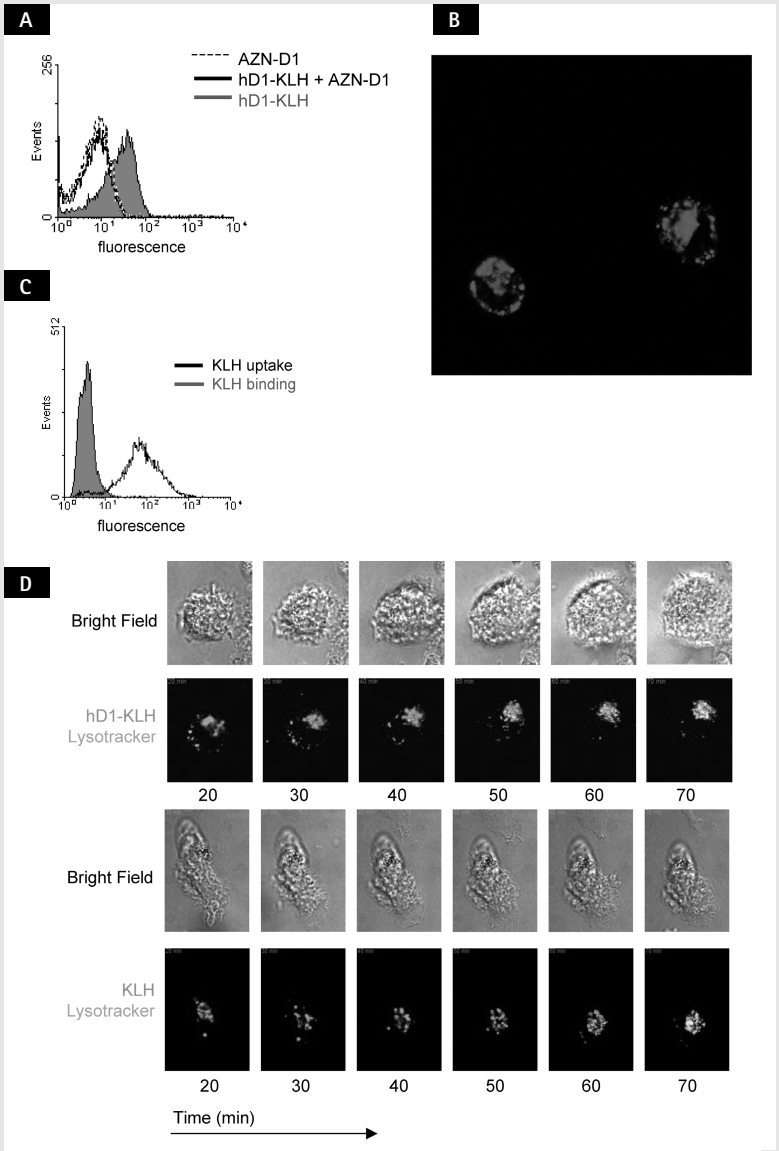


Figure 2

BINDING AND UPTAKE OF HD1-KLH BY DC. (A) iDC were treated with 10 µg/ml hD1-KLH, 100 µg/ml AZN-D1, or pretreated with 100 µg/ml AZN-D1 followed by 10 µg/ml hD1 incubation, followed by incubation with an Alexa Fluor 647-labeled goat anti-human IgG antibody. Binding of hD1-KLH was analyzed by flow cytometry. (B) Internalization of hD1-KLH was confirmed by CLSM. iDC were incubated with Alexa Fluor 647-labeled hD1-KLH (blue) for 1 h at 37°C, followed by microscopic analysis. The image represents the middle focal plane of the DC, with iris set at 2 nm. Original magnification, × 600. (C) iDC were incubated with Alexa Fluor 488-labeled KLH for 1 h at 4°C or 37°C. Subsequently, cells were fixed and analyzed by flow cytometric analysis. (D) iDC were labeled with LysoTracker Red, followed by addition of Alexa Fluor 488-labeled (green) hD1-KLH or KLH. Cells were imaged by CLSM. Data represent bright field and corresponding fluorescent images of cells at various time points after addition of hD1-KLH or KLH to the culture medium. Original magnification, × 630.



(data not shown). A time-course internalization experiment revealed that both the hD1 and the AZN-D1 antibodies were rapidly internalized by iDC, although the AZN-D1 antibody was internalized slightly more efficiently than hD1. As expected, the control AZN-L19 antibody, directed against CD18, was not internalized **(Fig. 1B)**. Analysis by confocal microscopy confirmed internalization of the hD1 and AZN-D1 antibodies by iDC, whereas the control antibodies 5G1.1, directed against human terminal complement protein C5, and mouse IgG1 isotype were not internalized **(Fig. 1C)**.

hD1-KLH is rapidly internalized and translocated to the lysosomal compartment

To further explore the potential of DC-SIGN as a targeting receptor for DC vaccination, we studied the uptake of KLH complexed to the humanized anti-DC-SIGN antibody hD1. As was observed with the hD1 antibody, hD1-KLH bound to iDC **(Fig. 2A)**. hD1-KLH binding to DC is mediated by DC-SIGN, as binding could be blocked by pretreating iDC with AZN-D1 **(Fig. 2A)**. Analysis by CLSM revealed that hD1-KLH was rapidly internalized by iDC **(Fig. 2B)**. After 1 h of incubation at 37°C, hD1-KLH could be detected in 74% of the iDC (data not shown). In contrast to hD1-KLH, KLH itself did not bind to iDC, but was internalized by iDC following 1 h incubation at 37°C **(Fig. 2C)**. These findings demonstrate that internalization of KLH by DC is not receptor-mediated, and likely depends on macropinocytosis.

Foreign antigens are taken up by DC and shuttled, via endosomes, to the lysosomal compartment where they can be processed. In order to establish whether hD1-KLH reaches the lysosomal compartment, and to study uptake-kinetics and routing of KLH and D1-KLH, these proteins were directly labeled with Alexa Fluor 488 dye. Live imaging by CLSM revealed rapid uptake of hD1-KLH and KLH by iDC. Both hD1-KLH and KLH were detected in the lysosomal compartment within 1 h after their addition to the culture medium **(Fig. 2D**; Supplemental Videos S1 and S2; see the Supplemental Videos link at the top of the online article, at the *Blood* website).

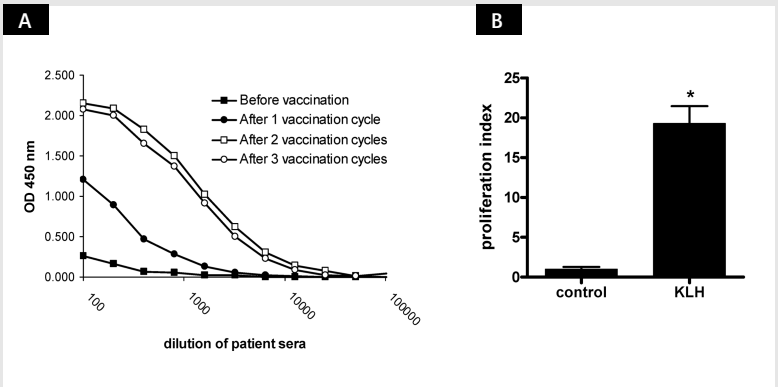
Induction of KLH responses by DC vaccination

In order to compare the antigen presentation capacity of DC pulsed with KLH to that of DC targeted by hD1-KLH, we required KLH-specific T cells. To this end, PBL were isolated from melanoma patients participating in a vaccination study. This study involved vaccination of patients with autologous DC pulsed *ex vivo* with KLH and melanoma-associated antigens. Monocyte-derived DC and PBL from patients showing humoral as well as cellular responses against KLH were used for the targeting studies.

All 31 vaccinated patients participating in the clinical vaccination trial showed responses against KLH (data not shown). Humoral responses were readily detected in the patients' serum. KLH-specific IgGs were present after the first vaccination cycle, and their serum levels increased upon the second vaccination **(Fig. 3A)**. Cellular responses were analyzed using PBMC obtained from patients one week before the start of the third vaccination cycle. Typically, PBMC pulsed with KLH showed an increased proliferative response compared to unpulsed PBMC, revealing the presence of KLH-reactive T cells in peripheral blood **(Fig. 3B)**. These findings show that KLH-pulsed mDC effectively induced both humoral and cellular responses *in vivo*.

Figure 3

HUMORAL AND CELLULAR RESPONSES AGAINST KLH IN PATIENTS VACCINATED WITH KLH-PULSED DC. (A) Patient serum was obtained both before the first, and after the first, second and third vaccination cycles. Total IgG antibodies specific for KLH were detected by ELISA. Data represent optical density (OD) 450 values of serially diluted serum samples for a representative patient. (B) Patient PBMC were isolated one week prior to the third vaccination cycle and cultured in the absence (control) or presence of 10 µg/ml KLH (KLH). Cellular responses were assessed in a tritiated thymidine incorporation assay. Data represent mean ± SD of experiments performed in 6-fold for a representative patient. Significant difference from control according to Student's *t*-test: $*p < 0{,}001$.



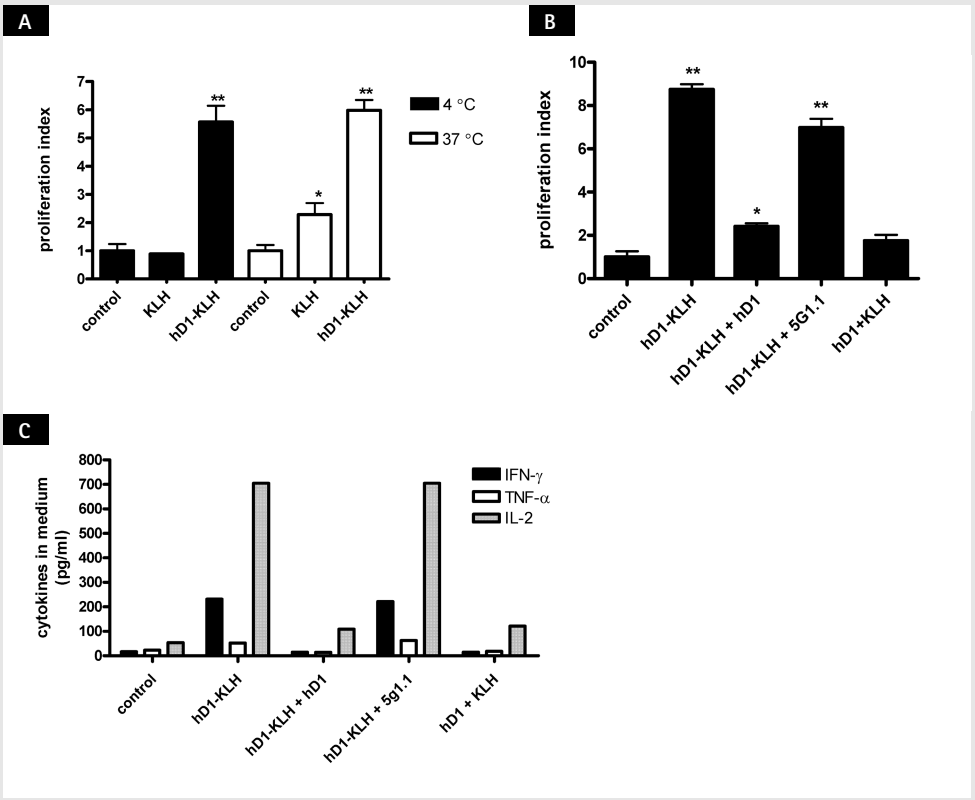
Targeting of KLH to DC-SIGN results in presentation of KLH antigen epitopes

To demonstrate that antibody-mediated targeting of antigen to DC-SIGN leads to antigen presentation, autologous iDC were incubated with 5 µg/ml KLH (± 0.63 nM) or hD1-KLH (± 0.53 nM) at 4°C for 1 h, washed to remove unbound protein, and added to PBL. Presentation of KLH epitopes to specific T cells was determined by measuring proliferative responses **(Fig. 4A, B)** and the levels of cytokines secreted in the medium **(Fig. 4C)**. As endocytotic processes are inhibited at 4 °C, and KLH does not bind to iDC **(Fig. 2C)**, hD1-KLH can only be internalized after binding to DC-SIGN. iDC incubated with hD1-KLH induced cellular responses by the PBL, whereas iDC incubated with KLH did not **(Fig. 4A, C)**, showing that targeting of KLH to DC-SIGN results in antigen presentation. We have previously demonstrated that the clinical vaccination protocol used in our studies results in a Th1-type immune response [43]. Indeed, stimulation of patient PBL with hD1-KLH treated iDC resulted in enhanced secretion of IL-2, IFN-γ and TNF-α **(Fig. 4C)**, while IL-4, IL-5 and IL-10 levels were not detectable (data not shown). As expected, iDC incubated with KLH at 37°C induced proliferative responses, as did iDC incubated with hD1-KLH **(Fig. 4A)**.

Presentation of KLH epitopes by iDC incubated with hD1-KLH at 4°C was abolished by preincubation of iDC with the hD1 antibody, but was unaffected by preincubation with the isotype control 5G1.1 **(Fig. 4B, C)**. This confirms that hD1-KLH targeted specifically to DC-SIGN, resulting in presentation of KLH epitopes. Cross-linking of hD1 to KLH was required for induction of DC-SIGN-mediated presentation of KLH epitopes, since iDC incubated with both KLH and hD1 antibody did not induce cellular responses **(Fig. 4B, C)**.

Figure 4

ANTIBODY-MEDIATED TARGETING OF ANTIGEN TO DC-SIGN RESULTS IN ANTIGEN PRESENTATION. (A) iDC were incubated with medium (control), 5 µg/ml KLH or 5 µg/ml hD1-KLH for 1 h at 4°C or 37°C. Subsequently, iDC were washed and cocultured with autologous KLH-responsive PBL, derived from the patients enrolled in the vaccination trial. After 4 days, cellular responses were assessed in a proliferation assay. Data are mean proliferation indices relative to medium control for experiments performed in triplicate ± SD. Significant difference from medium control according to ANOVA and Bonferroni test: *$p < 0.05$, **$p < 0.001$. (B) iDC were incubated with medium (control), 5 µg/ml hD1-KLH, 5 µg/ml hD1-KLH and 100 µg/ml hD1 (hD1-KLH + hD1), 5 µg/ml hD1-KLH and 100 µg/ml 5G1.1 (hD1-KLH + 5G1.1) or 5 µg/ml hD1 and 5 µg/ml KLH (hD1 + KLH) for 1 h at 4°C. Subsequently, iDC were washed and cocultured with autologous KLH-responsive PBL, derived from patients enrolled in the vaccination trial. After 4 days, cellular responses were assessed in a proliferation assay. Data are mean proliferation indices relative to medium control for experiments performed in triplicate ± SD. Significant difference from medium control according to ANOVA and Dunnett's test: *$p < 0.05$, **$p < 0.01$. (C) Production of IFN-γ, TNF-α and IL-2 in the coculture experiment described in (B). After 16 h of coculturing iDC and PBL supernatants were taken and cytokine levels were determined. Data represent cytokine levels in pooled samples of experiments performed in triplicate.



Targeting KLH to DC-SIGN enhances its immunogenicity

To determine whether DC-SIGN targeting increases the efficiency of antigen presentation, immature monocyte-derived DC from previously immunized patients were incubated at 37°C with various concentrations of KLH or hD1-KLH. Subsequently, iDC were washed, and a portion of the iDC was matured with LPS. hD1-KLH by itself did not induce maturation of iDC (data not shown). Both hD1-KLH treated iDC **(Fig. 5A)** and mDC **(Fig. 5B)** induced cellular responses at 100-fold lower concentrations than KLH-pulsed DC. Thus, antigen targeting via DC-SIGN significantly enhances immunogenicity.

KLH epitopes are presented up to 4 days after targeting DC-SIGN

After antigen uptake in the periphery, DC migrate to the draining lymph nodes to present the processed antigen to T cells. It is imperative that DC present the antigens over a prolonged period of time to initiate a significant immune response. We evaluated the efficiency of antigen presentation over time following targeting by incubating patient PBL with autologous iDC that had been pulsed with KLH or hD1-KLH 0, 2 or 4 days earlier. The results demonstrate that DC targeted with hD1-KLH presented KLH epitopes up to 4 days after targeting. Moreover, at all time points evaluated, DC targeted with hD1-KLH induced stronger proliferative responses than DC pulsed with KLH **(Fig. 6)**.

Figure 5

TARGETING OF ANTIGEN TO DC-SIGN ENHANCES ITS IMMUNOGENICITY. (A) iDC were incubated with medium, 5 µg/ml KLH or 5 µg/ml hD1-KLH for 1 h at 37°C. Subsequently, iDC were washed and cocultured with autologous KLH-responsive PBL, derived from patients enrolled in the vaccination trial. After 4 days, cellular responses were assessed in a proliferation assay. Data are mean proliferation indices relative to medium control for experiments performed in triplicate ± SD. (B) Experiment performed as described in (A), except that antigen-treated iDC were matured with LPS before addition of PBL.

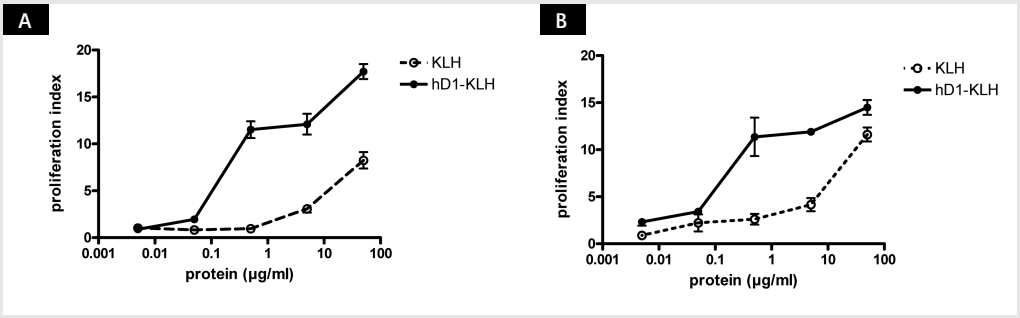


Figure 6

ANTIGEN TARGETED TO DC-SIGN IS PRESENTED FOR AT LEAST 4 DAYS. iDC were incubated with medium, 5 µg/ml KLH or 5 µg/ml hD1-KLH for 1 h at 37°C. Subsequently, iDC were washed and cultured for 0, 2 or 4 days before addition of autologous KLH-responsive PBL, derived from patients enrolled in the vaccination trial. Four days after addition of PBL, cellular responses were assessed in a proliferation assay. Data are mean proliferation indices relative to medium control for experiments performed in triplicate ± SD. Significant difference from KLH according to ANOVA and Bonferroni test: $*p < 0.05$, $**p < 0.01$.

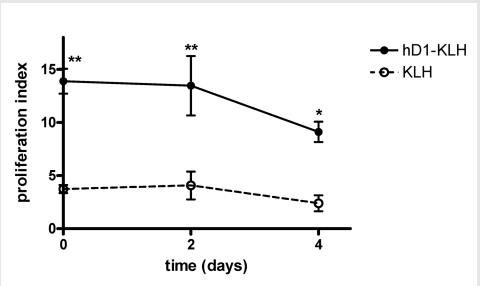
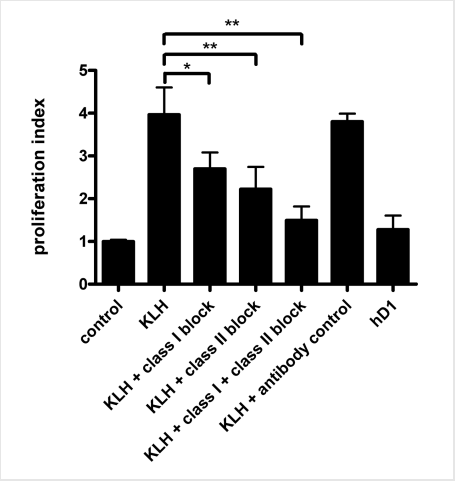


Figure 7

HD1-KLH TREATED DC ACTIVATE NAÏVE T CELLS RECOGNIZING KLH EPITOPES. iDC derived from a healthy donor were incubated with medium (control), 10 µg/ml KLH, or 10 µg/ml hD1 for 1 h at 37°C. Subsequently, cells were washed and matured with LPS. mDC were cocultured with autologous PBL that had been repeatedly stimulated with DC treated with hD1-KLH as described in "Material and methods". Some cocultures of KLH-pulsed mDC and PBL were supplemented with w6/32 antibody (KLH + class I block), IVA-12 (KLH + class II block), both w6/32 and IVA-12 (KLH + class I + class II block) or total mouse IgG (KLH + antibody control). Data are mean proliferation indices relative to medium control for experiments performed in triplicate ± SD. Significant difference according to ANOVA, followed by the Student-Newman-Keuls test: $*p < 0.01$, $**p < 0.001$.

Supplemental video 1. hD1-KLH and KLH are translocated to the lysosomes. iDC were labeled with LysoTracker Red and incubated with Alexa Fluor 488-labeled (green) hD1-KLH (A) or KLH (B). Uptake of hD1-KLH and KLH was studied by live imaging techniques. The time index in the top left-hand corner represents the time elapsed after addition of hD1-KLH or KLH to the culture medium.



hD1-KLH targeted to DC activates both MHC class I and II restricted naïve T cells recognizing KLH epitopes

To evaluate whether hD1-KLH, besides triggering recall responses, is able to activate naïve T cells, hD1-KLH was tested in an autologous *in vitro* culture system. PBL from a healthy donor were stimulated by repetitive coculturing with hD1-KLH-pulsed autologous DC. Il-10 was added to the cultures to stimulate expansion of $CD8^+$ T cells [44]. After 5 rounds of stimulation, PBL were cocultured with DC pulsed with KLH or hD1, to evaluate KLH-specific proliferative responses. The results demonstrate that hD1-KLH targeted DC activated KLH-specific naïve T cells **(Fig. 7)**. Presentation of KLH epitopes was mediated by MHC class I and class II, since both class I and class II blocking antibodies significantly reduced the proliferative response, while an antibody control did not. DC pulsed with hD1 antibody did not induce proliferative responses by the PBL **(Fig. 7)**, demonstrating that the T cell response is specific for KLH epitopes rather than epitopes contained within the hD1 antibody.

Discussion

In the present study we explored the capacity of the type II C-type lectin DC-SIGN to function as a target receptor for vaccination purposes. The results demonstrate that antibody-mediated targeting of antigen to human DC-SIGN was 100-fold more efficient than pulsing DC with antigen, and that antigen epitopes were presented for at least 4 days after targeting.

A critical step in designing a suitable targeting construct is the choice of targeting antibody. Various antibodies directed against the same receptor might bind distinct receptor epitopes, thus influencing biological outcome. For example, not all antibodies directed against DC-SIGN are internalized following binding [45], and the anti-MR antibody PAM-1 induces maturation of iDC, in contrast to a different isotype-matched anti-MR antibody [46]. The mouse anti-human DC-SIGN antibody AZN-D1 has been previously described to be internalized by DC [47], a prerequisite for successful targeting. However, the use of murine antibodies in humans presents numerous problems, including a short half-life and high immunogenicity [48]. We were able to graft the CDR of AZN-D1 hypervariable domains onto a human composite IgG2/IgG4 antibody without loss of binding characteristics. The resulting hD1 antibody exhibited a similar binding affinity for DC-SIGN as AZN-D1, and did not induce maturation of iDC (data not shown). hD1 binding induced rapid internalization of DC-SIGN, while internalization did not result in a lasting downregulation of DC-SIGN expression (data not shown), thus minimizing the effect a therapeutic intervention might have on the biological function of DC-SIGN. hD1 rapidly targeted antigen to the DC lysosomal compartment resulting in antigen presentation, and did not induce hD1-specific proliferative responses in the naïve T cell stimulation experiments. Thus, humanization of AZN-D1 resulted in an effective targeting antibody, and will facilitate introduction into clinical trials.

Vaccination strategies aimed at inducing cytotoxic T cell help, such as anti-tumor therapies, require antigen presentation in the context of MHC class I. In addition, the induction of CTL responses requires bystander $CD4^+$ T cell help [49]. Targeting DC with hD1-KLH resulted in the activation of naïve T cells in the context of both MHC class I and II. Although the major route for presentation of exogenous antigens is via class II, presentation via class I can occur via the process of cross-presentation. This process provides internalized proteins access to cytosolic proteasomes and their derived peptides access to the endoplasmatic reticulum (ER)-based class I processing machinery [50]. Particulate antigens taken up by phagocytosis have access to this machinery since phagosomes fuse with the ER soon after or during their formation [51]. Particulate antigens are more efficiently cross-presented than soluble antigens [52,53]. However, a recent study by Ackerman *et al.* reveals that internalized soluble proteins can escape proteolysis and also gain access to the lumen of the ER [54]. This might explain how antibody-mediated targeting of the type I C-type lectins MR [55] and DEC-205 [56] results in class I responses. Although we cannot exclude the possibility that, besides receptor-mediated endocytosis, a portion of our chimeric protein was taken up via macropinocytosis, our current findings strongly suggest that antibody-mediated targeting of DC-SIGN results in cross-presentation.

The technique commonly used in current clinical trials to load DC with antigens involves *ex vivo* incubation with MHC class I and II binding peptides. Other techniques involve loading with tumor lysates or apoptotic tumor cells,

and the introduction of genetic material to drive expression of specific antigens by the DC itself [57]. Ideally, vaccines should deliver antigens to the DC *in vivo*. Antibody-mediated targeting of antigens to DC surface receptors to stimulate antigen presentation *in vivo* has been shown to be far more potent than immunization with antigen in Complete Freund's Adjuvant or splenic DC pulsed with antigen *ex vivo* [58]. However, mere targeting of antigens to DC is not sufficient for induction of immunity. Antibody-mediated targeting of antigen to DEC-205 in mice leads to tolerance, and coadministration of a DC maturation stimulus is required to induce immunity [59-61]. These findings are consistent with DC-based vaccination studies in humans, showing that DC maturation is a prerequisite for induction of immunity [62]. Vaccination strategies for transplantation, allergy, autoimmunity and chronic inflammatory diseases could exploit the finding that targeted iDC induce tolerance. However, strategies aimed at inducing immunity will require a combination of antigens and DC activation factors. Agents that have been shown to activate DC *in vivo* include anti-CD40 antibody [63], α-galactosylceramide [64] and the Toll like receptor ligands LPS [65] and CpG oligonucleotides [66]. A better understanding of differences in DC subsets, their activation pathways and antigen uptake receptors will provide the information necessary for development of effective vaccines.

In the present study, we used a neo-antigen to study targeting efficiency. A major challenge in cancer therapy is to break immunologic tolerance to tumor-associated self antigens. Tolerance can be the result of clonal deletion, active suppression of antigen-specific T cells by regulatory T cells or inadequate activation stimuli provided by tumor cells upon antigen presentation, resulting in T cell anergy [67]. One way of breaking tolerance is by disruption of negative regulatory mechanisms directly at the T cell level. Antibody-mediated blockade of the cytotoxic T lymphocyte-associated antigen 4 (CTLA-4) breaks tolerance against moderately immunogenic tumors [68], and improves the efficiency of tumor cell-based vaccines against poorly immunogenic tumors [69]. Depletion of CD25+ regulatory T cells prior to blocking of CTLA-4 improves vaccine efficiency even further [70]. A second way of breaking tolerance is to recruit DC as potent APCs. DC can overcome the heightened threshold of anergic T cells [71] and restore responsiveness of tolerogenic tumor-specific T cells [72] *in vitro*. Moreover, studies in mice demonstrate that DC-based vaccines can break tolerance against (tumor-associated) self antigens [73-77] resulting in regression of established tumors [78]. Thus, a combination of antibody-mediated strategies targeting tumor-associated antigens to DC and strategies modulating regulatory mechanisms at the T cell level might provide effective cancer therapies.

In conclusion, antibody-mediated targeting of antigens to DC surface receptors represents an exciting way to induce immune responses. Our results demonstrate efficient delivery of antigen to DC via DC-SIGN, resulting in naïve as well as recall responses by T cells. These data expose DC-SIGN as a promising target molecule for antibody-mediated antigen delivery to DC.

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Chapter 8

Summary and Discussion

Summary and Discussion

DC play a central role in the regulation of the immune system. These cells can induce an effective immune response against pathogens, but in case of endogenous ligands DC have to induce tolerance to protect endogenous tissues from damage. This is a delicate balance dependent on the context in which the antigen is delivered to the DC. In case of a pathogenic antigen, 'danger' signals will be present that trigger the DC to mature and develop into a powerful antigen-presenting cell. These cells will activate T cells that specifically recognize the pathogen. In case of an endogenous ligand, no 'danger' signals are present rendering the DC immature and unable to activate specific T cells because of absence of a costimulatory signal. Therefore, immature DC induce tolerance against the endogenous ligand [1-3].
Besides activating or tolerizing T cells, DC also communicate with other cell types like B cells, NK cells, and neutrophils to coordinate adaptive and innate immune responses. Interactions with these cell types have implications on cell activation, antigen transfer and modulation of the type of immune response [4-6]. For instance, DC can transfer antigen to naïve B cells to initiate a specific antibody response [7] or activate NK cells in antiviral responses [4].
DC express the C-type II lectin DC-SIGN that recognize high-mannose and fucose moieties found on several pathogens, but also on some endogenous proteins. DC-SIGN can function both as antigen-uptake receptor as well as adhesion receptor and is implicated in several activities of a DC like antigen presentation [8], T cell activation [9], and DC migration [10]. The research described in this thesis focuses on the diverse implications of DC-SIGN in binding to foreign and endogenous ligands.

In **Chapter 1** a general introduction is given about the characteristics of C-type lectin receptors (CLRs) on DC. CLRs function as pattern recognition receptors (PRRs) by recognizing carbohydrate moieties on pathogens and thereby play an important role together with other PRRs in the immune defense against pathogens. Nevertheless, several pathogens like HIV-1 and *M. tuberculosis* exploit CLRs to escape from immune surveillance. In addition to these foreign ligands, CLRs can also recognize specific carbohydrate moieties on endogenous proteins and thereby mediate cell-cell adhesion, clearance of abnormal cells and plasma glycoprotein turnover. The CLR DC-SIGN is able to recognize both foreign as well as endogenous ligands and is involved in many aspects of the immune system. For instance, in **Chapter 2** it is shown that DC-SIGN can bind the fungus *C. albicans*. This was demonstrated using DC and DC-SIGN transfected cell-lines. Upon binding to DC-SIGN on immature DC, *C. albicans* was rapidly internalized into DC-SIGN enriched vesicles. These vesicles were distinct from vesicles containing mannose receptor which is another receptor for *C. albicans* on DC. Probably, the destiny of DC-SIGN$^+$ vesicles differs from mannose receptor$^+$ vesicles since the mannose receptor returns to the cell membrane whereas DC-SIGN ends up in the lysosomal compartments [8].
C. albicans is harmless under normal conditions, but can lead to severe infections in the immunocompromised host [11,12]. Induction of cell-mediated immunity is crucial in the host defense against *C. albicans*. DC play an important role in this cell-mediated induction by processing and presentation of *C. albicans* to T cells [13]. The specific interaction of *C. albicans* to

DC-SIGN expressed on DC and subsequent internalisation suggest a role in this cell-mediated immunity. Likely, this is the most important role for DC in interacting with *C. albicans* as its anticandidal capacity is quite weak in relation to monocytes and macrophages as demonstrated in **Chapter 3.** *C. albicans* can present itself in two forms: blastoconidia, the yeast form and hyphae considered as the filamentous form [14]. Besides intracellular killing of blastoconidia, also extracellular damage to hyphae induced by DC was lower than that of monocytes and macrophages. Moreover, in response to *C. albicans*, DC also produced less TNF-α, IL-6 and IL-8 compared to monocytes and macrophages. Interestingly, also differences in cytokine production were observed between monocytes and macrophages. Monocytes released greater amounts of cytokines upon stimulation with blastoconidia compared to hyphae whereas the opposite was found for macrophages. These cells produced more TNF-α and IL-8 upon stimulation with the hyphal form. This suggests that monocytes and macrophages have adapted to encounter a specific phenotypic form of *C. albicans*.

Originally, DC-SIGN was discovered as adhesion molecule in DC-T cell interactions [9]. It was hypothesised that DC-SIGN-ICAM-3 interactions are the first interactions to be established between DC and T cells followed by other adhesive molecules like LFA-1-ICAM-1. However, its role in T cell activation became controversial as several studies did not show a role for DC-SIGN in T cell activation whereas other studies did [15-18]. Therefore, in **Chapter 4** the relevance of DC-SIGN in DC-induced T cell proliferation is studied. Numerous mixed lymphocyte reactions (MLR) were performed and it was observed that anti-DC-SIGN antibodies only blocked T cell proliferation in a weak MLR. Consequently, as mature DC are more potent APC than immature DC, hardly any effect with MLR driven by mature DC was seen. This indicates an initial role for DC-SIGN that is dominated by other adhesive and costimulatory mechanisms. Interestingly, LFA-1 was dependent on the strength of the T cell response as well. With the use of recombinant DC-SIGN we observed that approximately 5% of PBL could bind to DC-SIGN. This was not attributed to one specific population but comprised all major lymphocyte subsets. There was variation among donors with a maximum of 20% binding. PBL of one donor showed a high DC-SIGN binding capacity and the MLR with these cells was more prone to DC-SIGN blocking antibodies compared to a donor whose PBL had a low DC-SIGN binding capacity. In the MLR of the donor whose PBL have a high DC-SIGN binding capacity, DC-SIGN can bind firmly to a higher percentage of T cells and may have a prolonged effect on these cells compared to cells that bind transiently. Therefore, an effect by anti-DC-SIGN in MLR may be observed more rapidly.

In **Chapter 5** we discovered that the DC-SIGN coated beads bound a substantial higher percentage of monocytes than PBL. Previously, van Gisbergen *et al.* [19] showed that soluble DC-SIGN could bind neutrophils but not monocytes. These authors showed that the binding of DC-SIGN to neutrophils occurs via Lewis X which is not present on monocytes [19]. However, a low Lewis X expression was observed when using another anti-Lewis X antibody. Likely, the DC-SIGN beads can engage simultaneous interactions with several spatially dispersed unsialylated Lewis X epitopes on monocytes because of its large interaction surface (1 μm diameter beads). This results in a stable binding to monocytes whereas soluble DC-SIGN can not establish a stable binding to monocytes because of a smaller contact surface and therefore the number

of interacting molecules remains low. However, the inability of soluble DC-SIGN to bind monocytes is overcome when the number of Lewis X epitopes is increased by neuraminidase treatment of monocytes. Neuraminidase removes terminal sialic residues of various glycomolecules. The resulting increased Lewis X expression indicates that Lewis X on monocytes is masked by sialyl groups. Although the physiological significance of DC-SIGN interacting with monocytes remains to be determined, it is tempting to speculate about a role in DC-monocyte communication via DC-SIGN-Lewis X interactions.

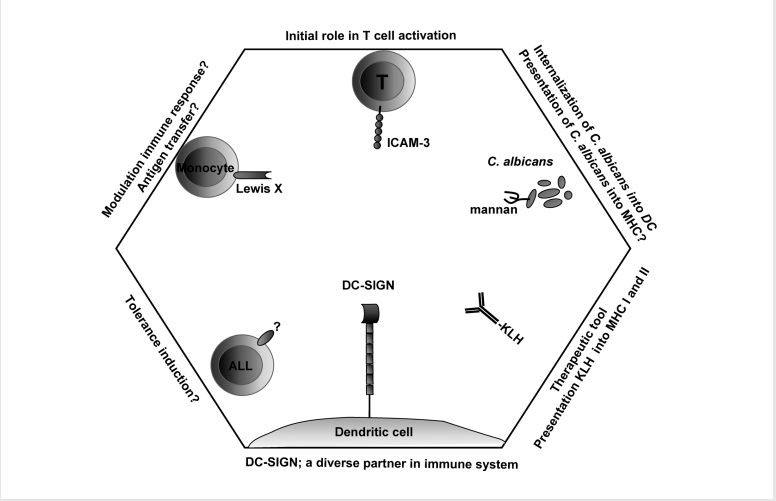
Glycosylation is frequently altered in tumor cells when compared to their normal counterparts. An aberrant glycosylation pattern can be beneficial for the tumor cell because it enables metastasis or protection from immune surveillance [20]. In **Chapter 6** we studied whether DC-SIGN and its homologue L-SIGN could detect aberrant glycosylation on leukemic cells that might be of prognostic relevance. DC-SIGN and L-SIGN coated beads showed an increased binding to most acute lymphoblastic leukaemia (ALL) cells compared to cells from healthy donors. DC-SIGN did not discriminate between B- and T-ALL cells whereas L-SIGN bound preferentially to B-ALL bone marrow cells. T-ALL can be subdivided into immature T-ALL, common T-ALL, and mature T-ALL according to the chronological development of T cells [21,22]. This revealed a preference of DC-SIGN for binding to mature T-ALL. B-ALL can be subdivided into pro-B-ALL, common B-ALL, and pre-B-ALL [22], but this yielded no clear differences in binding to DC-SIGN or L-SIGN. Although Lewis X is a likely binding partner for DC-SIGN in tumor cells [23], only tumor cells of some B-ALL patients showed a high expression of this carbohydrate. Therefore, ligands other than Lewis X on most ALL cells will bind to DC-SIGN. Interestingly, increased binding of peripheral blood leukemic cells to DC-SIGN and L-SIGN is correlated with a poor survival compared to increased binding of bone marrow cells to DC-SIGN and L-SIGN. Possibly, once leukemic cells enter the circulation interactions with DC-SIGN and L-SIGN positive cells are possible. As DC-SIGN and L-SIGN are expressed on cells that can induce tolerance (liver-sinusoidal endothelial cells, DC) it supports a role in favouring immune escape of the leukemic cells. Future studies are required to study the potential of developing a therapeutic tool.

Chapter 7 describes the potential of using DC-SIGN as target molecule for DC-vaccination strategies. Current DC-based vaccination strategies are based on *ex vivo*-generated autologous DC loaded with an antigen prior to readministration into patients [24]. Targeting antigens to DC *in vivo* via DC-SIGN would be a more direct and less laborious way. For this purpose, a humanized DC-SIGN antibody (hD1) was chemically cross-linked to a model antigen keyhole limpet hemocyanin (KLH) resulting in the chimeric antibody-protein complex hD1-KLH. hD1-KLH specifically bound to DC-SIGN and was internalized and translocated to the lysosomal compartment of DC. DC targeted with hD1-KLH induced memory T cell responses against KLH at 100-fold lower concentration than DC targeted with KLH alone. This suggests that targeting KLH to DC-SIGN enhances its immunogenicity.

Moreover, targeting DC with hD1-KLH resulted in the activation of naïve T cells in the context of MHC I and II molecules. This indicates activation of both $CD4^+$ and $CD8^+$ T cells which is necessary in an effective anti-tumor vaccination therapy. The presentation of KLH epitopes in MHC I suggests the occurrence of cross-presentation along the major route for presentation of exogenous antigens via MHC II [25]. Targeting DC with hD1-KLH four days

Figure 1

DIVERSE IMPLICATIONS OF DC-SIGN IN BINDING TO ITS LIGANDS. Because of its carbohydrate specificity DC-SIGN can interact both with pathogen-derived as well as endogenous glycoconjugates. Interactions with its different ligands result in involvement of DC-SIGN in several aspects of immune system like antigen-presentation and cellular communication.



earlier before contact with T cells still resulted in a specific anti-KLH T cell response. This is very important as *in vivo* after antigen uptake in the periphery it will take time for a DC to reach the lymph node. Therefore it is crucial that a DC can present antigen for a prolonged time to induce an efficient immune response. Altogether, these results demonstrate that DC-SIGN is a promising target molecule for antibody-mediated antigen delivery to DC.

In conclusion, this thesis demonstrates that DC-SIGN is involved in many aspects of the immune system **(Fig. 1)**. It plays a role in immune defense and antigen presentation by taking up *C. albicans* and the targeting construct hD1-KLH. Moreover, this C-type lectin can act as adhesion receptor by mediating T cell activation and binding to monocytes and leukemic cells. These diverse functions are in line with other DC-SIGN ligands like ICAM-2 for DC migration [10], Lewis X on Mac-1 and CEACAM for communication with neutrophils [26], and a large array of pathogens (reviewed in [27]) for immune defense and escape mechanisms. Additionally, interaction of DC-SIGN with tumor cells has also been demonstrated recently [23]. All these various DC-SIGN ligands have in common that they are specifically glycosylated since DC-SIGN recognizes highly branched-mannose- and fucose-containing glycans [28,29]. The expression of these specific glycans is regulated by specific glycosyltransferases that catalyse the synthesis of oligosaccharides [30]. The expression of human glycosyltransferases can be tissue specific and can depend on differentiation status of the cell and the presence of cytokines [31-34]. Therefore, glycosylation can change in cancer cells [20] and activated immune cells like monocytes that upregulate Lewis X expression on their cell surface upon activation by LPS or pro-inflammatory cytokines [35,36]. Possibly, DC-SIGN plays an important role in these modified glycosylation circumstances as it can bind tumor cells, monocytes and neutrophils.

The functional consequences upon interaction of DC-SIGN with monocytes and tumor cells is not known yet, but can be extrapolated from its interactions with neutrophils and pathogens. For instance, interaction of DC with activated neutrophils via DC-SIGN results in maturation of the DC [19]. This mechanism is expected to be used by activated monocytes as well.
Many pathogens exploit DC-SIGN to evade immunity. For example, *M. tuberculosis* interacts with DC-SIGN on DC and thereby inhibits the immunostimulatory function of DC by producing the immune response dampening cytokine IL-10 [37]. Moreover, the human gastric pathogen *H. pylori* modulates the function of DC via DC-SIGN by blocking the polarization towards a T helper type 1 response [38]. Also several viruses like HIV-1, HCV and Measles virus exploit DC-SIGN for their dissemination [39-41].
Whether interaction of DC-SIGN with *C. albicans* may result in immune evasion is not known yet. It seems unlikely, as DC-SIGN preferentially bound the blastoconidia form of *C. albicans* to which a protective response (Th1 type) is initiated by DC [42]. Interestingly, DC-SIGN hardly bound the hyphal form of *C. albicans* (unpublished results from A. Cambi) to which DC initiate a non-protective response (Th2 type) [42]. Possibly, DC-SIGN plays a role in induction of Th1 response against the conidia form of *C. albicans* in a similar way as reported for the IgtB polysaccharide from *N. meningitides* [43]. This glycosylated structure binds to DC-SIGN and skews DC immune responses towards Th1 helper activity [43].
Nevertheless, DC-SIGN seems to be a popular molecule for most pathogens to escape from immune surveillance and this might be the case for cancer cells as well. In support of this, there are some indications that tumor carbohydrates affect DC via C-type lectins [44,45]. For instance, the tumor-associated antigen MUC1 can induce monocytes to differentiate into tolerogenic DC [45]. In addition, MUC1 prevents development of a Th1 type anti-tumor response upon binding to DC [44]. Possibly, DC-SIGN internalizes specific tumor antigens in the absence of danger signals resulting in tolerance of T cells specific for the tumor antigen in a similar way as described for DEC-205 [46]. However, tolerance induction is overcome by addition of a strong DC maturation stimulus [47]. Therefore, in future patient tumor vaccination studies a DC maturation stimulus should be included together with our DC-SIGN targeting construct (Chapter 7) to induce an effective anti-tumor response. Interestingly, the fact that targeting an antigen to a C-type lectin can either induce antigen-specific immune activation or tolerance dependent on the presence or absence of a DC maturation stimulus provides a promising therapeutic tool to be applied not only in cancer, but also in infectious- and autoimmune diseases.
Targeting DC-SIGN results in rapid internalisation of DC-SIGN, but this internalisation did not result in a lasting down-regulation of DC-SIGN expression. Such a temporary down-regulation of DC-SIGN expression minimizes its effect on the biological function of DC-SIGN like T cell activation and communication with neutrophils and monocytes. In support of this, we did not observe an effect on T cell proliferation in the targeting experiments. Nevertheless, the role of DC-SIGN in T cell activation is not very robust as its effect is only visible in weak MLR and strikingly only a small number of T cells can bind to DC-SIGN beads. Apparently, DC-SIGN mediates a subtle role in DC-T cell communication which might be involved in tolerance induction as well since DC-SIGN is able to stimulate or down-regulate T cell activation dependent on the strength of T cell stimulus [18].
It is becoming clear that besides T cells, DC can communicate with several

types of immune cells to coordinate adaptive and innate immune responses [48]. Our binding data suggest a role for DC-SIGN in communication between DC and monocytes (Chapter 5), B cells and NK(T) cells (Chapter 4). Several reports describe a role for DC with B cells [5,7,49] and NK(T) cells [50-52], however as far as we know communication of DC with monocytes has not been reported before. Possibly, monocytes and DC meet each other in inflamed peripheral lymph nodes since large numbers of monocytes can be recruited to these sites [53]. There, bi-directional cross-talk between DC and monocytes likely results in an effective innate and adaptive immune response against the invaded microorganisms. This can be established by several mechanisms like maturation of DC, activation of monocytes, antigen transfer, and modulation type of adaptive immune response (Th1 or Th2). It will be interesting to study whether DC-SIGN just serves as an adhesion receptor to bring the cells together or whether it is also involved in the possible mechanisms described above. Also its function in DC communication with B cells and NK(T) cells requires further attention.

Although most interest goes to DC-SIGN on DC, there are several reports in which expression of DC-SIGN is detected on other cells like macrophages [54], B cells [55], liver sinusoidal endothelium [56], platelets and megakaryocytes [57,58]. Possibly, interaction of DC-SIGN to several ligands and its related functional outcomes are more explicitly developed in these cell types. For instance, leukemic cells may predominantly interact with DC-SIGN on LSEC instead of DC to induce tolerance against the leukemic cells or to mediate clearance of these malignant cells from the circulation [59,60]. Also, interaction of DC-SIGN to monocytes may be physiologically relevant when this interaction takes place between monocytes and platelets in promoting atherogenesis and thrombosis [61]. Future studies are required to analyze the function of DC-SIGN on these cell types in depth, but the fact that DC-SIGN is also expressed on cells other than DC further supports its diverse role in the immune system.

Altogether, the various ligands of DC-SIGN show that this C-type lectin is involved in many aspects of the immune system. The different outcomes upon interaction with DC-SIGN can be explained by its organization into microdomains [62] in which associated molecules may collaborate to determine the outcome of the interacting ligand.

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Nederlandse Samenvatting

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Dendritische cellen (DC) spelen een belangrijke rol in de regulatie van het immuunsysteem. Deze cellen kunnen een krachtige afweerreactie opwekken tegen ziekteverwekkers, maar in het geval van lichaamseigen stoffen dienen DC tolerantie te induceren om de lichaamseigen weefsels te beschermen tegen schade. De uiteindelijke reactie van de DC hangt af van de samenhang waarin het antigeen aan de DC wordt aangeboden. In het geval van een ziekteverwekker zijn er 'gevaar' signalen aanwezig die de DC aanzetten tot verdere rijping en ontwikkelt de DC zich tot een krachtige antigeenpresenterende cel. Deze cellen zullen de specifieke T cellen, die uitsluitend gericht zijn op de ziekteverwekker, activeren. In het geval van een lichaamseigen stof zijn er geen 'gevaar' signalen aanwezig waardoor de DC 'immature' (onrijp) blijft en de specifieke T cellen niet kan activeren door de afwezigheid van een costimulatie signaal. Daardoor induceren immature DC tolerantie tegen de lichaamseigen stof.
Naast het instrueren van T cellen tot activering of tolerantie, communiceren DC ook met andere cel types zoals B cellen, NK cellen, en neutrofielen om de adaptieve en aangeboren immuunreacties te coördineren. Communicatie met deze cel types heeft gevolgen voor cel activering, antigeen overdracht, en sturing van het type immuunreactie. Zo kunnen DC antigenen overdragen naar rustende B cellen om een specifieke antistofreactie op te wekken of door NK cellen te activeren in antivirale reacties.
De C-type II lectine DC-SIGN wordt tot expressie gebracht op DC en herkent complexe mannose en fucose structuren die aanwezig zijn op ziekteverwekkers, maar ook op sommige lichaamseigen eiwitten. DC-SIGN kan zowel als antigeen-opname receptor als adhesie receptor functioneren en is betrokken in verschillende activiteiten van een DC zoals antigeen presentatie, activering van T cellen, en DC migratie. Het onderzoek beschreven in dit proefschrift richt zich op de verschillende aspecten van DC-SIGN in de binding aan lichaamsvreemde en lichaamseigen stoffen.

In **Hoofdstuk 1** wordt een algemene introductie gegeven over de kenmerken van C-type lectine receptoren (CLRs) op DC. CLRs functioneren als patroonherkennings receptoren (PRRs) door het herkennen van suikerstructuren op ziekteverwekkers en spelen daardoor een belangrijke rol samen met andere PRRs in de immuunafweer tegen ziekteverwekkers. Toch zijn er verscheidene ziekteverwekkers zoals HIV-1 en *Mycobacterium tuberculosis* die de CLRs uitbuiten om te ontsnappen aan eliminatie door het immuunsysteem. Naast deze lichaamsvreemde stoffen, kunnen CLRs ook suikerstructuren herkennen op lichaamseigen eiwitten en spelen dan een rol bij cel-cel adhesie, opruiming van abnormale cellen en verwijdering van geglycosyleerde plasma eiwitten. De CLR DC-SIGN kan zowel lichaamsvreemde als lichaamseigen stoffen herkennen en is betrokken bij veel aspecten van het immuunsysteem. Zo wordt in **Hoofdstuk 2** aangetoond dat DC-SIGN aan de schimmel *Candida albicans* kan binden. Deze binding werd gemeten door gebruik te maken van DC en cellijnen die kunstmatig DC-SIGN tot expressie brengen. Na binding aan DC-SIGN op immature DC werd *C. albicans* snel geïnternaliseerd in DC-SIGN positieve 'vesicles' (blaasjes). Deze vesicles verschilden van mannose receptor$^+$ vesicles, een andere receptor voor *C. albicans* op DC. Mogelijk verschilt het lot van de DC-SIGN$^+$ vesicles met die van mannose receptor$^+$ vesicles aangezien de mannose receptor een receptor is die terug-

keert naar de celmembraan terwijl DC-SIGN in lysosomale compartimenten eindigt.
C. albicans is ongevaarlijk in normale omstandigheden, maar kan leiden tot ernstige infecties in personen met een verzwakte immuunrespons. Het opwekken van cellulaire immuniteit is cruciaal in de afweer van de gastheer tegen *C. albicans*. DC spelen een belangrijke rol in deze cellulaire immuniteit door het verwerken en presenteren van *C. albicans* aan T cellen. De specifieke interactie van *C. albicans* met DC-SIGN op de DC en aansluitende internalisatie maakt een rol in deze cellulaire immuniteit erg aannemelijk. Waarschijnlijk is dit de meest belangrijke rol voor DC in zijn interactie met *C. albicans* aangezien zijn mogelijkheden om *C. albicans* te doden beperkt zijn in vergelijking met monocyten en macrofagen zoals aangetoond in **Hoofdstuk 3.** *C. albicans* kan voorkomen in twee vormen: 'blastoconidia', de gistvorm en 'hyphae', de draadvorm. Naast intracellulaire doding van de blastoconidia vorm, is ook de extracellulaire schade aan hyphae toegebracht door DC kleiner dan die van monocyten en macrofagen. Verder produceerden DC ook minder cytokines zoals TNF-α, IL-6 en IL-8 vergeleken met monocyten en macrofagen in reactie op *C. albicans*. Interessant genoeg waren er ook verschillen in cytokine productie tussen monocyten en macrofagen. Monocyten scheidden grotere hoeveelheden cytokines uit na stimulatie met blastoconidia vergeleken met hyphae terwijl het tegenovergestelde werd gevonden voor macrofagen. Deze cellen produceerden meer TNF-α en IL-8 na stimulatie met de hyphae vorm. Dit suggereert dat monocyten en macrofagen zich aangepast hebben om een specifieke verschijningsvorm van *C. albicans* aan te pakken.

DC-SIGN werd ontdekt als adhesiemolecuul in DC-T cel interacties. Er werd gespeculeerd dat de DC-SIGN-ICAM-3 interacties de eerste interacties zijn tussen DC en T cellen die opgevolgd worden door andere adhesie moleculen zoals LFA-1-ICAM-1. De rol van DC-SIGN in T cel activering werd controversieel, omdat in een aantal studies geen rol voor DC-SIGN in T cel activering werd beschreven terwijl andere studies dit wel beschreven. In **Hoofdstuk 4** is daarom de relevantie van DC-SIGN in DC-geïnduceerde T cel proliferatie onderzocht. Er werden meerdere 'mixed lymphocyte reactions' (MLR) uitgevoerd en er kwam naar voren dat anti-DC-SIGN antistoffen alleen de T cel proliferatie blokkeerden in een zwakke MLR. Daar 'mature' (rijp) DC betere antigeen-presenterende cellen zijn dan immature DC, was er geen effect te zien in een MLR die door mature DC werd geïnduceerd. Dit geeft een initiële rol aan voor DC-SIGN die overgenomen wordt door andere adhesie en costimulatoire mechanismen. Interessant genoeg was de invloed van LFA-1 ook afhankelijk van de sterkte van de T cel reactie. Met het gebruik van recombinant DC-SIGN zagen we dat ongeveer 5% van de perifere bloed lymfocyten (PBL) aan DC-SIGN kon binden. Dit bleek niet één specifieke populatie, maar bevatte alle lymfocyten subpopulaties. Er werd variatie gevonden in het percentage binding door de donoren met een maximum van 20% binding. PBL van één donor liet een hoge DC-SIGN bindingscapaciteit zien en de MLR met deze cellen bleek gevoeliger voor DC-SIGN blokkerende antistoffen vergeleken met een donor wiens PBL een lage DC-SIGN bindingscapaciteit had. In de MLR van de donor die PBL heeft met een hoge DC-SIGN bindingscapaciteit kan DC-SIGN een hoger percentage van T cellen binden op een stabiele manier waardoor het een langer effect kan hebben op deze cellen vergeleken met cellen die vluchtig binden. Daarom wordt een effect met anti-DC-SIGN in MLR waarschijnlijk sneller gezien.

In **Hoofdstuk 5** staat beschreven dat DC-SIGN gekoppeld aan 'beads' (bolletjes) een veel hoger percentage monocyten kon binden dan PBL. Eerder is door Van Gisbergen *et al.* aangetoond dat DC-SIGN moleculen neutrofielen kunnen binden maar geen monocyten. Deze auteurs toonden aan dat de binding van DC-SIGN aan neutrofielen plaatsvindt via Lewis X. Deze suiker is niet aanwezig op monocyten. Er werd echter een lage expressie van Lewis X op monocyten waargenomen wanneer een ander anti-Lewis X antistof werd gebruikt. Waarschijnlijk kunnen de DC-SIGN gekoppelde beads gelijktijdig interacties aangaan met verschillende ver verspreide Lewis X epitopen op monocyten vanwege zijn grote interactie oppervlakte (1 µm diameter). Dit resulteert in een stabiele binding aan monocyten terwijl vrije DC-SIGN moleculen geen stabiele binding aan monocyten kunnen bewerkstelligen vanwege een kleiner contact oppervlakte waardoor het aantal moleculen die een interactie met elkaar aangaan laag blijft. De beperking van vrije DC-SIGN moleculen om monocyten te binden wordt overwonnen als het aantal Lewis X epitopen wordt verhoogd door een neuraminidase behandeling van de monocyten. Neuraminidase verwijdert eindstandige sialyl groepen van verschillende geglycosyleerde eiwitten. De resulterende verhoogde Lewis X expressie geeft aan dat Lewis X op monocyten gemaskeerd wordt door sialyl groepen. Hoewel de fysiologische betekenis van DC-SIGN binding aan monocyten nog bepaald moet worden is het aannemelijk om te speculeren over een rol in DC-monocyt communicatie via DC-SIGN-Lewis X interacties.

De glycosylering is vaak veranderd in kankercellen in vergelijking met hun normale tegenhangers. Een afwijkende glycosylering kan gunstig zijn voor een kankercel omdat het metastasering of ontsnapping aan eliminatie door het immuunsysteem mogelijk maakt. In **Hoofdstuk 6** werd onderzocht of DC-SIGN en zijn homoloog L-SIGN afwijkende glycosyleringspatronen op leukemische cellen kunnen detecteren die mogelijk van prognostische waarde zijn. DC-SIGN en L-SIGN gekoppeld aan beads bonden beter aan de meeste acute-lymfoblastaire leukemische (ALL) cellen vergeleken met cellen van gezonde donoren. DC-SIGN bond evengoed aan B- als T-ALL cellen terwijl L-SIGN bij voorkeur B-ALL beenmerg cellen bond. T-ALL kan onderverdeeld worden in 'immature' T-ALL, 'common' T-ALL, en 'mature' T-ALL op basis van de chronologische ontwikkeling van T cellen. Een voorkeur voor DC-SIGN voor binding aan 'mature' T-ALL werd waargenomen. B-ALL kan onderverdeeld worden in 'pro-B-ALL', 'common' B-ALL, en 'pre-B-ALL', maar dit resulteerde niet in duidelijke verschillen in binding aan DC-SIGN of L-SIGN. Hoewel Lewis X een bindingspartner kan zijn voor DC-SIGN in kankercellen, vertoonden de kankercellen van slechts een paar B-ALL patiënten een hoge expressie van deze suikerstructuur. Daarom zullen andere liganden dan Lewis X op de meeste ALL cellen aan DC-SIGN binden. Een interessante bevinding was dat verhoogde binding van perifere bloed leukemische cellen aan DC-SIGN en L-SIGN gerelateerd is met een slechtere overlevingskans vergeleken met verhoogde binding van beenmergcellen aan DC-SIGN en L-SIGN. Waarschijnlijk zijn interacties met DC-SIGN en L-SIGN mogelijk zodra de leukemische cellen de bloedcirculatie binnendringen. Het feit dat DC-SIGN en L-SIGN tot expressie worden gebracht op cellen die tolerantie kunnen opwekken (lever sinusoidale endotheelcellen, DC) ondersteunt een rol in het bevorderen van ontsnapping van leukemische cellen aan het immuunsysteem. Vervolgstudies zijn nodig om de mogelijkheid te onderzoeken voor het ontwikkelen van een therapeutisch middel.

Hoofdstuk 7 beschrijft de mogelijkheid voor het gebruik van DC-SIGN als doelwit molecuul voor DC vaccinatie. Huidige DC-gebaseerde vaccinatie methodes zijn gebaseerd op *ex vivo*-gekweekte eigen DC die beladen zijn met een antigeen alvorens ze toegediend worden aan patiënten. Het richten van antigenen naar DC *in vivo* via DC-SIGN zou een veel directere en minder bewerkelijke methode kunnen zijn. Om de mogelijkheid hiervan te testen werd een gehumaniseerd DC-SIGN antistof (hD1) chemisch gekoppeld aan het model antigeen keyhole limpet hemocyanin (KLH) wat resulteert in de chimere antistof-eiwit complex: hD1-KLH. hD1-KLH bond specifiek aan DC via DC-SIGN en werd geïnternaliseerd en overgeheveld naar het lysosomale compartiment van DC. DC die zo werden beladen met hD1-KLH waren in staat om een geheugen T cel reactie tegen KLH te induceren bij een 100-maal lagere concentratie dan KLH alleen. Dit suggereert dat het richten van KLH naar DC-SIGN zijn immunogeniciteit verhoogt.
Verder resulteerde het aanbieden van hD1-KLH aan DC in de activering van rustende T cellen in de context van de speciale presentatie moleculen MHC I en II. Dit betekent dat zowel $CD4^+$ als $CD8^+$ T cellen worden geactiveerd wat nodig is voor een effectieve anti-tumor vaccinatie therapie. De presentatie van KLH epitopen in MHC I geeft aan dat naast de gebruikelijke route van presentatie van oplosbare antigenen via MHC II ook ´cross`-presentatie heeft opgetreden. Het beladen van DC met hD1-KLH vier dagen eerder voor het contact met T cellen resulteerde nog steeds in een effectieve anti-KLH T cel reactie. Dit is erg belangrijk aangezien na antigeen opname *in vivo* in de periferie, het enige tijd duurt voordat een DC de lymfe knoop bereikt. Daarom is het cruciaal dat een DC antigenen kan presenteren over langere periodes om een effectieve immuunreactie te starten. Samenvattend laten deze resultaten zien dat DC-SIGN een veelbelovend doelwit is voor het aanbieden van antigeen aan DC door middel van een antistof.

List of Abbreviations
Dankwoord
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List of Abbreviations

ALL	Acute lymphoblastic leukaemia
APC	Antigen-presenting cell
(m)Ab	(monoclonal) antibody
BM	Bone marrow
BSA	Bovine serum albumin
Ca^{2+}	Calcium
CD	Cluster of differentiation
CFSE	Carboxyfluorescein diacetate succinimidyl ester
CLRs	C-type lectin receptors
CLSM	Confocal laser scanning microscopy
CRD	Carbohydrate recognition domain
DC	Dendritic cell(s)
DC-SIGN	DC-specific ICAM-3 grabbing non-integrin
ELISA	Enzyme-linked immunosorbent assay
FACS	Fluorescence activated cell sorter
FCS	Fetal calf serum
GM-CSF	Granulocyte/macrophage colony stimulating factor
ICAM	Intercellular adhesion molecule
IFN	Interferon
Ig	Immunoglobulin
IL	Interleukin
KLH	Keyhole limpet hemocyanin
LFA-1	Leukocyte functional antigen-1
LPS	Lipopolysaccharide
LSEC	Liver sinusoidal endothelial cells
L-SIGN	Liver/lymph node SIGN
MFI	Mean fluorescence intensity
MHC	Major histocompatibility complex
MLR	Mixed lymphocyte reaction
MR	Mannose receptor
NK	Natural killer
PAMPs	Pathogen-associated molecular patterns
PB	Peripheral blood
PBL	Peripheral blood lymphocytes
PBMC	Peripheral blood mononuclear cells
PBS	Phosphate-buffered saline
PRRs	Pattern recognition receptors
RT	Room temperature
TCR	T cell receptor
TEM	Transmission electron microscopy
Th	T helper
TLRs	Toll like receptors
TNF	Tumor necrosis factor

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Karlijn

Curriculum Vitae

Karlijn Gijzen werd geboren op 11 oktober 1978 te Alkmaar. In 1997 behaalde zij haar eindexamen Atheneum aan het Bernardus Alfrink College te Schagen en begon in datzelfde jaar aan de studie Gezondheidswetenschappen aan de Universiteit van Maastricht. Tijdens deze studie koos ze als afstudeerrichting Biologische Gezondheidkunde. Aan de Universiteit van Maastricht liep ze stage op de afdeling Farmacologie en Toxicologie bij Dr. GR Haenen, Dr. GM den Hartog en Dr. CG Heijnen en deed onderzoek naar flavonoïden. Als afsluiting van de studie deed ze onderzoek naar de angiogenese remmer β-pep25 (heden Anginex) bij de afdeling Tumor Angiogenese Laboratorium aan de Universiteit van Maastricht onder begeleiding van Prof. Dr. AW Griffioen, Dr. DWJ van der Schaft en Dr. JCA Bouma-ter Steege. Met de scriptie van dit afstudeeronderzoek heeft ze de Catherina Pijls aanmoedigingsprijs gewonnen die uitgereikt werd door de Universiteit van Maastricht. In augustus 2001 slaagde ze voor het doctoraal examen en begon in januari 2002 als Junior Onderzoeker op de afdeling Tumor immunologie van het UMC St. Radboud te Nijmegen bij Prof. Dr. CG Figdor en Dr. R Torensma. De resultaten van dat promotieonderzoek staan beschreven in dit proefschrift. Sinds december 2006 is ze werkzaam als postdoc bij de afdeling Vaccin Onderzoek van het Nederlands Vaccin Instituut te Bilthoven.

List of Publications

Cambi A, **Gijzen K**, de Vries IJM, Torensma R, Joosten B, Adema GJ, Netea MG, Kullberg BJ, Romani L, Figdor CG. The C-type lectin DC-SIGN (CD209) is an antigen-uptake receptor for *Candida albicans* on dendritic cells. *Eur J Immunol. 2003 Feb;33(2):532-8*.

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